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REVIEW

Pasteurella multocida: Genotypes and Genomics

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SUMMARY *Pasteurella multocida* is a highly versatile pathogen capable of causing infections in a wide range of domestic and wild animals as well as in humans and nonhuman primates. Despite over 135 years of research, the molecular basis for the myriad manifestations of *P. multocida* pathogenesis and the determinants of *P. multocida* phylogeny remain poorly defined. The current availability of multiple *P. multocida* genome sequences now makes it possible to delve into the underlying genetic mechanisms of *P. multocida* fitness and virulence. Using whole-genome sequences, the genotypes, including the capsular genotypes, lipopolysaccharide (LPS) genotypes, and multilocus sequence types, as well as virulence factor-encoding genes of *P. multocida* isolates from different clinical presentations can be characterized rapidly and accurately. Putative genetic factors that contribute to virulence, fitness, host specificity, and disease predilection can also be identified through comparative genome analysis of different *P. multocida* isolates. However, although some knowledge about genotypes, fitness, and pathogenesis has been gained from the recent whole-genome sequencing and comparative analysis studies of *P. multocida*, there is still a long way to go before we fully understand the pathogenic mechanisms of this important zoonotic pathogen. The quality of several available genome sequences is

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low, as they are assemblies with relatively low coverage, and genomes of *P. multocida* isolates from some uncommon host species are still limited or lacking. Here, we review recent advances, as well as continuing knowledge gaps, in our understanding of determinants contributing to virulence, fitness, host specificity, disease predilection, and phylogeny of *P. multocida*.

KEYWORDS *Pasteurella multocida*, virulence, fitness, host specificity, disease predilection, phylogeny, whole-genome sequencing, comparative genomic analysis

INTRODUCTION

The genus *Pasteurella* was named in honor of the famous French microbiologist and chemist Louis Pasteur, who first isolated this bacterium in the 1880s as the causative agent of "*choléra des poules*" (fowl cholera) (1). Since that time, *Pasteurella* species have been linked as causative agents of many infectious diseases in a wide range of animals as well as in humans (reviewed in references 2 and 3). Like most *Pasteurella* species, *Pasteurella multocida* is often found as part of the normal oropharyngeal microbiota of many animals and birds but can also be a primary or opportunistic pathogen of the upper and lower respiratory tracts (described in reference 4). Three subspecies of *P. multocida* were formally recognized in 1985, *P. multocida* subsp. *multocida*, subsp. *gallicida*, and subsp. *septica* (5).

P. multocida is a small, nonmotile, facultatively anaerobic, Gram-negative coccobacillus, measuring approximately 0.3 to 1.0 μm in width and 1.0 to 2.0 μm in length (described in references 4 and 6). Typical bipolar staining with methylene blue can be seen in smears taken from wounds or tissues. *P. multocida* strains can be cultured on rich medium, such as tryptic soy agar, chocolate agar, or brain heart infusion agar, supplemented with 5 to 10% fetal calf serum or horse or sheep blood, under aerobic conditions at 37°C; they are oxidase and catalase positive; and they can ferment many carbohydrates, such as fructose, glucose, mannose, and galactose, to produce acid but not gas (described in references 4 and 7). Biochemical tests can be used for preliminary differentiation of *P. multocida* subspecies, where *P. multocida* subsp. *multocida* isolates are generally dulcitol negative and sorbitol positive, *P. multocida* subsp. *gallicida* isolates are generally dulcitol positive and sorbitol negative, and *P. multocida* subsp. *septica* isolates are generally dulcitol negative and sorbitol negative (8). Molecular techniques such as ribotyping based on sequence analysis of the 16S rRNA gene, repetitive extragenic palindromic sequence PCR (REP-PCR), and/or multilocus sequence typing (MLST) analyses can more easily and accurately differentiate subspecies of *P. multocida* (9). For example, using ribotyping, a putative novel subspecies, designated *P. multocida* subsp. *tigris*, was identified from a human infection resulting from a tiger bite wound (10). More recently, MLST analysis further differentiated isolates from wild chimpanzees that displayed conflicting fermentation patterns such that they could not be assigned to *P. multocida* subsp. *gallicida* or subsp. *septica*; the MLST analysis revealed that the isolates were more closely related to *P. multocida* subsp. *gallicida* (11).

P. multocida is capable of infecting a wide spectrum of domestic and wild mammals, birds, and reptiles as well as humans (reviewed in reference 3). The most common *P. multocida*-associated diseases of economic importance to global animal husbandry are fowl cholera in avian species, hemorrhagic septicemia (HS) and bovine respiratory disease in ruminant species, progressive atrophic rhinitis and pneumonic pasteurellosis in swine, and snuffles in rabbits (described in reference 2). In humans, opportunistic infections resulting from bite or scratch wounds are relatively common, especially in elderly and immunocompromised individuals (described in reference 2), although chronic pulmonary infection in humans can result from exposure to mucous secretions from animals or pets with respiratory colonization or infection (reviewed in reference 3). In addition, clinical cases of urinary tract infection (12, 13) and bacteremic meningitis have been reported (14). Human deaths due to *P. multocida* infection are uncommon, with reports in the United States ranging from 2 to 25 between 1993 and 2006 (reviewed in reference 3) and even fewer more recently (15).

Despite more than 135 years of study, *P. multocida* remains an enigmatic pathogenic bacterium. Until recently, only incremental progress had been made toward understanding the molecular basis of *P. multocida* pathogenesis and host disease predilection. However, whole-genome sequences of a number of *P. multocida* strains have now become available, which enables the application of new approaches to study the underlying genetic mechanisms associated with virulence, fitness, and host specificity of *P. multocida* (16–23). In this review, we provide a summary of the molecular insights into the genotypes, fitness, and pathogenesis of *P. multocida* that have been gained from recent whole-genome sequencing (WGS) and comparative analysis studies. We also note areas where gaps in our knowledge remain.

GENOTYPING AND GENOTYPES

Traditionally, *P. multocida* isolates have been serologically classified into five capsular serogroups (A, B, D, E, and F) and 16 lipopolysaccharide (LPS) serovars (serovar 1 through serovar 16), using methods developed by Carter in 1952 (7, 24, 25) and Heddleston et al. in 1972 (26), respectively. These serological typing methods have been widely used in epidemiological studies to determine the serovars of *P. multocida* strains that are circulating in different host species and that are associated with different types of diseases. For example, strains associated with fowl cholera are most frequently determined to be of serovar A:1, A:3, or A:4 (27); HS is commonly associated with serovars B:2 and/or E:2 (28); bovine respiratory diseases are associated with serovar A:3 (reviewed in reference 29); progressive atrophic rhinitis in swine is generally induced by toxigenic serogroup D and/or A strains (30); and pasteurellosis in rabbits is generally associated with serovar A:3, A:4, or A:12 (31, 32). However, these two serological typing methods require high-quality antisera, which are difficult to prepare and implement for clinical use (described in references 4 and 33). Therefore, these serological methods are not commonly used any longer for typing *P. multocida* isolates in many laboratories. Instead, more rapid, accurate, and reproducible molecular sequence typing methods have become more popular for diagnostics.

The most frequently used molecular typing methods for *P. multocida* are capsular genotyping, LPS genotyping, MLST, and/or virulence genotyping based on the detection of different virulence gene profiles (34–36). The capsular genotyping method was developed based on PCR detection of *cap* genes, using primers highly specific for different serogroups: *hyaD-hyaC* for serogroup A, *bcbD* for serogroup B, *dcbF* for serogroup D, *ecbJ* for serogroup E, and *fcbD* for serogroup F (37). The genotypes determined by this multiplex capsular PCR-based typing system reliably and accurately discriminate among the capsular serogroups. For example, this method was used to determine the capsular genotypes of *P. multocida* isolates from different host species from different geographic regions and displaying different disease symptoms (36, 38–44). Results from these epidemiological studies suggest that capsular genotypes A, D, and F are commonly associated with fowl cholera, conjunctivitis, and respiratory disorders such as rhinitis, pneumonia, and shipping fever (36, 38, 40, 44–46), while genotypes B and E are more frequently associated with HS (41, 47). Early publications documented that capsular type F is commonly seen in avian species, especially from turkeys that have fowl cholera (2, 48), but this capsular genotype is also detected in nonavian hosts such as swine and rabbits (20, 40, 49), where it is highly pathogenic to these hosts (20, 50). Nontypeable strains (not serogroups A, B, D, E, and F) have also been found in pneumonic cases (36, 51). Although the capsular genotyping method has many advantages, including being rapid and providing identification even when serotyping fails, compared to the traditional serological method, the capsular genotyping method is not a phenotypic test and may give a positive result for some strains that do not actually produce a capsular polysaccharide as a result of mutation (4).

While *P. multocida* strains produce LPS with a variable outer core (52), they share only eight unique LPS outer core biosynthesis loci (Fig. 1). For example, LPS serovar 1 and 14 strains share the type L1 outer core biosynthesis locus; serovar 2 and 5 strains share the type L2 locus; serovar 3 and 4 strains share the type L3 locus; serovar 6 and

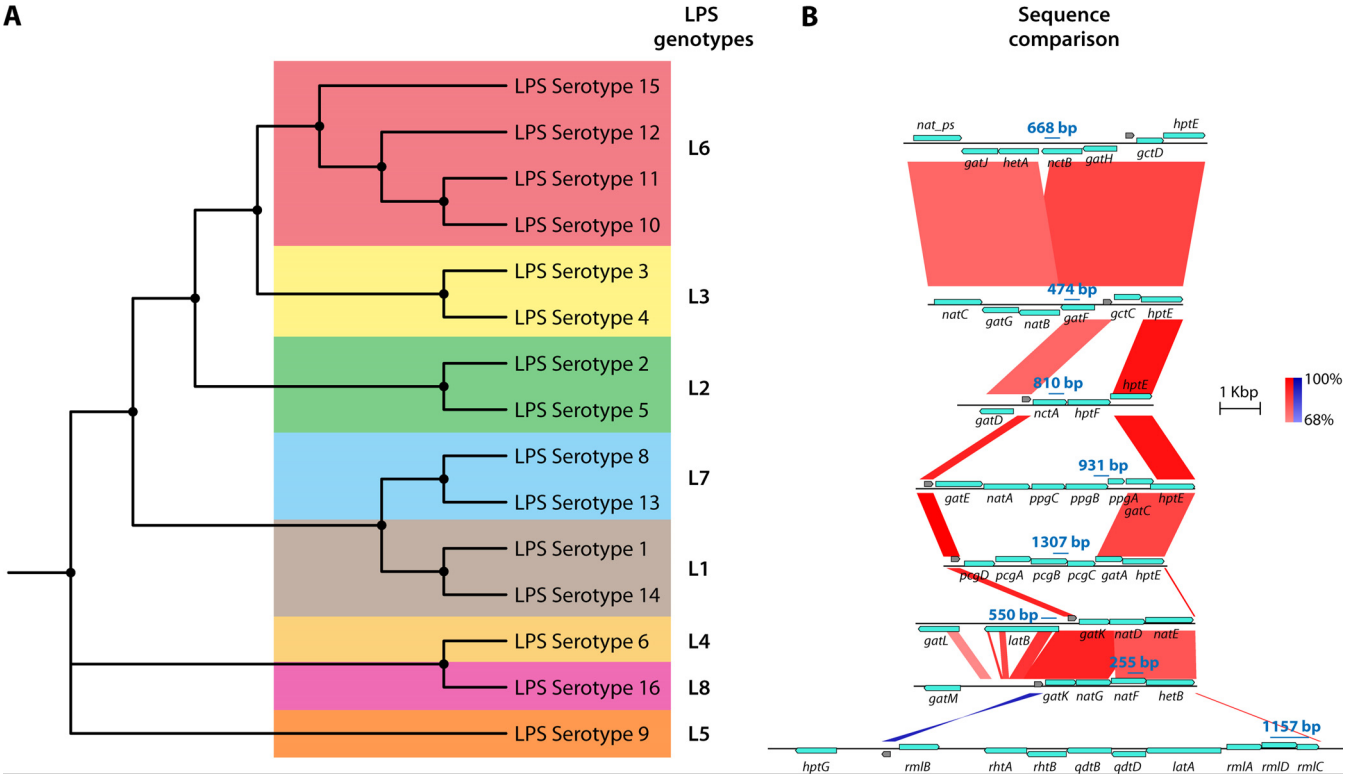

FIG 1 Genetic organization of the LPS outer core biosynthesis loci found in 16 LPS serotype strains of *Pasteurella multocida*. (A) Phylogenetic tree of 16 *P. multocida* strains with different LPS serotypes, constructed based on the DNA sequences of the LPS outer core biosynthetic genes. The gene organizations of the corresponding *P. multocida* LPS outer core loci with different LPS serotypes are shown. (B) Comparative analysis of the LPS outer core-encoding loci among different LPS serotypes. Shown are synteny plots of the corresponding LPS serotypes in panel A, visualized by EasyFig 2.2.3 (155). Arrows (cyan) denote the genes and their direction within the locus. Color-coded shading denotes the BLASTn identity of the regions between genomes. The relative position and size (base pairs) of each genotype-specific PCR amplicon are shown as bars above each LPS outer core biosynthesis locus.

7 strains share the type L4 locus; serovar 9 strains share the type L5 locus; serovar 10, 11, 12, and 15 strains share the type L6 locus; serovar 8 and 13 strains share the type L7 locus; and serovar 16 strains share the type L8 locus (53–59). A multiplex PCR assay, based on the genetic organization of the LPS outer core biosynthesis loci, enabled the differentiation of the 16 LPS serovar strains of *P. multocida* into eight genotypes (L1 to L8) (60). This LPS-based genotyping method provides a rapid and effective way to determine *P. multocida* LPS genotypes for epidemiological studies. One such study revealed that the most dominant LPS genotype associated with swine respiratory disorders in China appears to be L6, followed by L3 (36). Both of these two genotypes are also the main LPS genotypes of *P. multocida* isolates recovered from diseased swine in Australia (43). The LPS genotypes of isolates from other mammals with different diseases have also been found to be mostly L6, followed by L3, including field isolates of *P. multocida* from sheep with respiratory diseases in Iran (61), lung isolates from dead alpacas in Peru (62), and leporine isolates involved in infections in Italy (40). These findings suggest that L6 and L3 might be the dominant patho-LPS genotypes for infections in mammals. In avian *P. multocida*, the LPS genotypes seem to be more heterogeneous. The isolates from fowl cholera in Australia were determined to be of L3, L1, L4, and L6 (43), while only L1 and L3 were found for the isolates from fowl cholera in southwest China (38). While the LPS genotyping method has a noted advantage in being able to identify strains more rapidly, even when the traditional serotyping method fails, the results from LPS genotyping do not always correspond to the observed LPS serovars (60), and it may even give an apparent positive result for some strains that do not produce an intact LPS or produce a modified LPS, as a result of mutation or acquisition of a gene encoding an LPS-modifying enzyme.

TABLE 1 Documented *P. multocida* capsular:LPS(:MLST) genotypes and their associated diseases

Type of pasteurellosis	Capsular:LPS genotype(s)	Capsular:LPS:MLST genotype(s)	Reference
Avian fowl cholera	A:L1, A:L3	A:L1:RIRDC ST129, A:L1:RIRDC ST342, A:L3:RIRDC ST8	38
Porcine respiratory diseases	A:L3, A:L6, D:L6, F:L3	A:L3:ST3, A:L3:multihost ST74, A:L6:multihost ST10, D:L6:multihost ST10, D:L6:multihost ST11, D:L6:multihost ST16, D:L6:multihost ST75, F:L3:multihost ST12	36
Bovine HS	B:L2	B:L2:RIRDC ST122	19
Leporine pasteurellosis	A:L3, A:L6, D:L6, F:L3	A:L3:RIRDC ST9, A:L3:RIRDC ST24, A:L3:RIRDC ST204, A:L3:RIRDC ST294, A:L3:RIRDC ST304, A:L3:RIRDC ST312, A:L6:RIRDC ST74, A:L6:RIRDC ST297, A:L6:RIRDC ST300, A:L6:RIRDC ST301, A:L6:RIRDC ST302, D:L6:RIRDC ST50, D:L6:RIRDC ST293, D:L6:RIRDC ST296, D:L6:RIRDC ST305, D:L6:RIRDC ST311, D:L6:RIRDC ST313, F:L3:RIRDC ST9, F:L3:RIRDC ST295, F:L3:RIRDC ST301, F:L3:RIRDC ST310	40

An MLST method has been developed by Rural Industries Research and Development Corporation (RIRDC) for genotyping of avian isolates of *P. multocida*, based on the conserved regions of seven housekeeping genes: *adk*, *est*, *gdh*, *mdh*, *pgi*, *pmi*, and *zwf* (63). Although the RIRDC-based MLST method was initially designed for genotyping of avian isolates, it is used widely to type *P. multocida* isolates from nonavian species (40, 43, 47, 64, 65). In addition to the RIRDC-based MLST scheme, another MLST scheme (multihost MLST scheme) was developed based on comparative nucleotide sequence analysis of fragments from the seven housekeeping enzyme genes *adk*, *aroA*, *deoD*, *gdhA*, *g6pd*, *mdh*, and *pgi* to investigate evolutionary relationships among bovine, ovine, porcine, and avian isolates of *P. multocida* (66). This multihost MLST scheme has been used in epidemiological studies (34, 36). Both of these MLST schemes are available to the public at the *Pasteurella multocida* MLST website (http://pubmlst.org/pmultocida/). Since the RIRDC MLST database currently has more genotypes (365 genotypes [RIRDC sequence type 1 {ST1} to RIRDC ST365], as of 31 May 2019) than the multihost MLST database (109 genotypes [ST1 to ST109], as of 31 May 2019), some researchers opt to use the RIRDC MLST scheme to type *P. multocida* isolates from nonavian species (40).

In most studies, there is a good correlation between the MLST genotypes and the types of diseases observed. For instance, previous studies have shown that the multihost MLST genotypes ST3 (RIRDC ST13), ST10 (RIRDC ST74), and especially ST11 (RIRDC ST50) are strongly associated with swine respiratory disease in China (36). The multihost MLST genotype ST11 is also prevalent in rabbits from Spain and Italy, where it is strongly associated with pneumonia (34, 40, 67). In addition, isolates with multihost MLST genotype ST72 (RIRDC ST129) were strongly associated with fowl cholera in China (38, 68), while isolates with multihost MLST genotype ST44 (RIRDC ST122) were associated with HS in Southeast Asia (47).

Considering the importance of capsule and LPS for virulence and host specificity of *P. multocida*, the application of genotyping methods that combine the capsular, LPS, and MLST genotyping systems would be useful for characterization of clinical isolates to study the global epidemiological and molecular evolutionary aspects of this pathogen. While such tools are not yet available, several studies have characterized *P. multocida* isolates by combining the capsular genotypes and the LPS genotypes as well as the MLST genotypes (19, 36). These studies point to a notable correlation of specific types of diseases with certain capsular:LPS genotypes and/or capsular:LPS:MLST genotypes (Table 1). For instance, they found that porcine respiratory diseases, such as progressive atrophic rhinitis and pneumonic pasteurellosis, are mainly associated with capsular:LPS genotypes D:L6, A:L3, and A:L6 and/or capsular:LPS:MLST genotypes D:L6:ST11 (RIRDC ST50), A:L6:ST10 (RIRDC ST74), and A:L3:ST3 (RIRDC ST13) in China (36). Data from other studies showed that the genotypes of isolates from avian fowl cholera are strongly associated with capsular:LPS genotype A:L1 or capsular:LPS:MLST genotype A:L1:ST72 (RIRDC ST129) (38), while isolates from pasteurellosis in rabbits in Italy are mainly associated with capsular:LPS genotypes A:L6, D:L6, and F:L3 and/or capsular:

LPS:MLST genotype F:L3:ST12 (RIRDC ST9), D:L6:ST11 (RIRDC ST50), or A:L6:ST10 (RIRDC ST74) (40).

P. multocida genomes contain many genes encoding putative virulence factors that contribute to the pathogenesis of *P. multocida* (17, 23, 69), so virulence genotyping based on the detection of different virulence gene profiles has also been developed and used as a useful genotyping method (36, 39, 40, 51, 70–73). In addition to capsule- and LPS-associated genes, virulence genes commonly chosen for virulence genotyping in epidemiological studies include those encoding fimbriae and other adhesins (*ptfA*, *fimA*, *hsf-1*, *hsf-2*, *pfhA*, and *tadD*), toxin (*toxA*), iron acquisition proteins (*exbB*, *exbD*, *tonB*, *hgbA*, *hgbB*, *fur*, and *tbpA*), sialidases (*nanB* and *nanH*), hyaluronidase (*pmHAS*), outer membrane proteins (OMPs) (*ompA*, *ompH*, *oma87*, and *plpB*), and superoxide dismutase (*sodA* and *sodC*) (36, 39, 40, 51, 70–73). Some of these genes, such as *ptfA*, *fimA*, *hsf-2*, *exbB*, *exbD*, *tonB*, *fur*, *sodA*, *sodC*, *ompA*, *ompH*, *oma87*, and *plpB*, are broadly characteristic of *P. multocida*, so they have a high detection rate among isolates of different genotypes and/or from different host species (36, 39, 40, 51, 70–73) (Fig. 2). However, some virulence genes are detected in only a limited number of isolates and so are primarily associated with specific types of diseases and/or certain host species. For instance, *toxA* is found only in strains associated with progressive atrophic rhinitis, and *tbpA* is found only in isolates of bovine and ovine sources (36, 44, 69) (Fig. 2).

More recently, WGS data of *P. multocida* have been applied for assigning capsular, LPS, and MLST genotypes (19). DNA sequences for determining different genotypes are conveniently available through the *Pasteurella multocida* MLST website (http://pubmlst.org/pmultocida/). It is also possible to rapidly identify all of the known and putative virulence genes within a given sequenced genome by comparative analysis using the WGS data publicly available through the NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/?term=Pasteurella+multocida). For example, a recent study was able to discriminate the capsular, LPS, and RIRDC MLST genotypes among *P. multocida* isolates from different geographic regions and from different hosts manifesting different clinical diseases (Fig. 3) (19). In this study, the capsular:LPS:RIRDC MLST genotype B:L2:ST122 was associated with bovine HS, while the capsular:LPS:RIRDC MLST genotype D:L6:ST50 was associated with swine respiratory disease. These results agree with the other clinical epidemiological data obtained through PCR analysis (34–36, 45, 46, 51, 63, 68, 74). On the other hand, there appeared to be a limited correlation between the genotypes of the *P. multocida* isolates and the corresponding hosts or disease presentations. That is, although genotype B:L2:ST122 was frequently associated with bovine HS, another isolate with this genotype, HN04 (GenBank accession no. PPVE00000000), was recovered from swine HS (19). In other examples, the capsular:LPS:RIRDC MLST genotype D:L6:ST50 was found associated with both progressive atrophic rhinitis and pneumonia in swine (36), while the capsular:LPS:RIRDC MLST genotype F:L3:ST9 was recovered from both swine (20) and chickens (75). It is possible that more obvious congruence will emerge once additional genomes and their metadata become available.

SEQUENCED *P. MULTOCIDA* GENOMES IN GENBANK

Until recently, the generation of multiple *P. multocida* genome sequences lagged behind those of many other Gram-negative pathogenic bacteria. As of 31 December 2018, there are 176 whole-genome sequences for *P. multocida*, including 47 complete sequences and 129 draft sequences (comprised of 2 to 3,154 scaffolds) publicly available through the NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/?term=Pasteurella+multocida). Many of these genomes are sequenced and assembled using next-generation sequencing (NGS) or second-generation platforms such as the Illumina platforms and not through single-molecule sequencing (SMS) or third-generation technologies such as the PacBio or the Nanopore platform. However, the read lengths achieved by NGS platforms are limited, compared to those of the SMS platforms (reviewed in reference 76). In addition, for some of the draft genomes, a

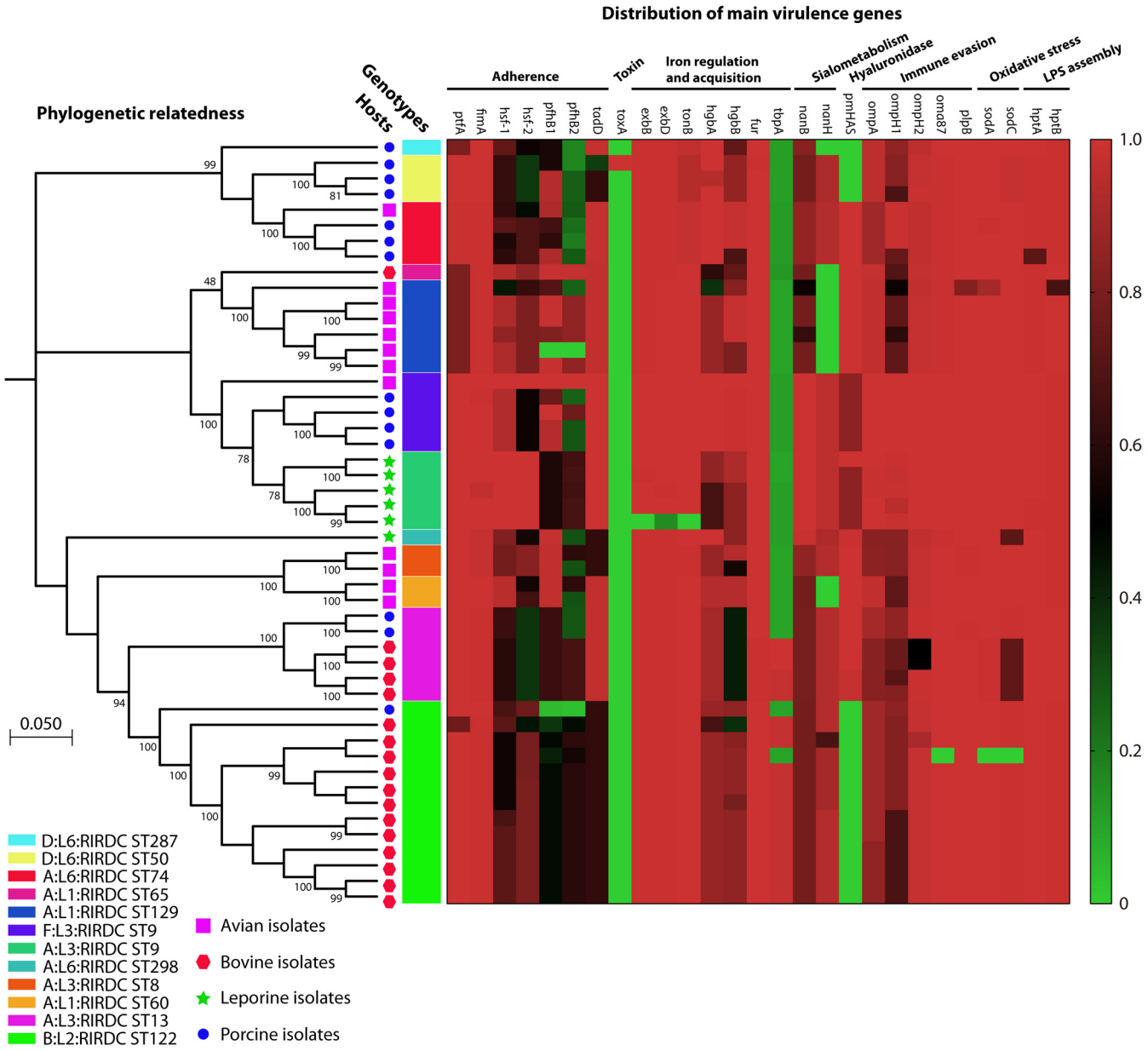

FIG 2 Distribution of the main virulence genes found among *P. multocida* isolates of different genotypes and/or from different host species. Shown is the phylogenetic relationship of 49 *P. multocida* genome sequences from 12 avian isolates, 17 bovine isolates, 14 swine isolates, and 6 leporine isolates (left dendrogram). Also shown is a heat map of virulence genes (center columns) found among the *P. multocida* isolates of different genotypes (left column) or from different host species (color-coded in the key). A BLAST score ratio (BSR) analysis was performed on these virulence genes. The presence of a virulence gene in a genome was determined based upon the BSR analysis with a normalized ratio of ≥0.8 (156).

number of the genes used for genotyping fall within the gaps not covered by the contigs. Therefore, the quality of some of the *P. multocida* draft genomes or assemblies currently available (as of 31 December 2018) might not be as high as desirable.

Most of the sequenced genomes possessed only a single circular chromosome. However, four of the genomes, HN06 (GenBank accession no. CP003313), CIRMBP-0884 (GenBank accession no. CP020345), FDAARGOS_218 (GenBank accession no. CP020405), and S298D (GenBank accession no. CM009574), included an additional plasmid (GenBank accession no. CP003314, CP020346, CP020404, and CM009574, respectively), while another genome, CIRMBP-0873 (GenBank accession no. CP020347), possessed two plasmids (GenBank accession no. CP020348 and CP020349).

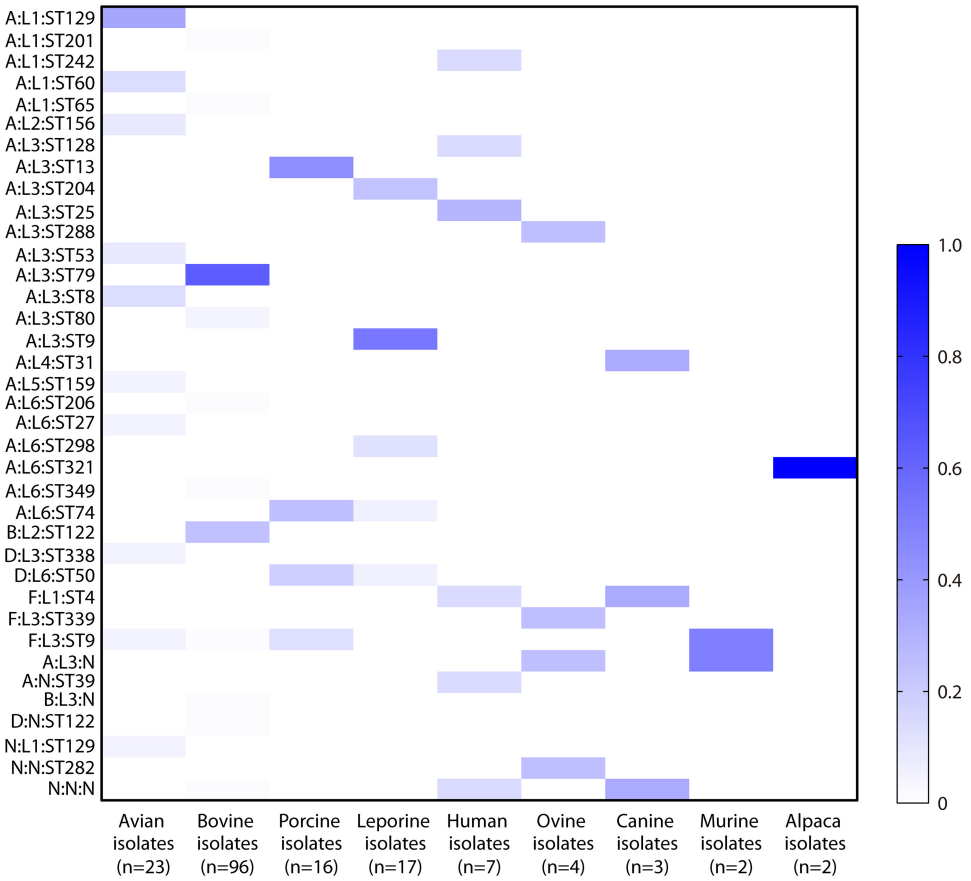

FIG 3 Heat map showing the capsular:LPS:RIRDC MLST genotypes of *P. multocida* strains from different host species. Genotyping was performed by using the whole-genome sequences of 170 *P. multocida* strains for which host information is available in the NCBI database (as of 31 December 2018). Blocks in shaded color denote the scaled distributions of each genotype among *P. multocida* isolates from a specific host species.

With the exception of five strains, NCTC10382 (GenBank accession no. LS483473), NCTC10722 (GenBank accession no. UGST01000000), NCTC11620 (GenBank accession no. UGSW01000000), NCTC11995 (GenBank accession no. UGSV01000000), and NCTC8489 (GenBank accession no. UGSU01000000), for which no host information is available and one sequence derived from metagenomic reads (UBA9365; GenBank accession no. DMUM01000000), the remaining 170 isolates originated from bovine species, avian species, leporine species, porcine species, humans, ovine species, canine species, murine species, and alpacas (Fig. 4A). It should be noted that three strains, BS168 (GenBank accession no. CP031554), EB168 (GenBank accession no. CP031553), and TB168 (GenBank accession no. CP031552), are derived from the 168th culture passage of strain PM8-1 (GenBank accession no. CP031551), which was continuously passaged *in vitro* in broth containing no antibiotics or different types of antibiotics (enrofloxacin or ceftriaxone).

Overall, the whole-genome size, average percent G+C content, and predicted numbers of genes and encoded putative proteins harbored by the different *P. multocida* strains were similar (Table 2 and Fig. 4A). The 176 sequenced genomes from different hosts (Fig. 4Ba) possessed 4 categories of capsular genotypes (A, B, D, and F) (Fig. 4Bb), 7 categories of LPS genotypes (Fig. 4Bc), and 36 categories of MLST genotypes (Fig. 4Bd). Several draft genomes were nontypeable, presumably due to gaps present between genome contigs in the assemblies, where some of the genes used for capsular/LPS/MLST genotyping fell within these gaps.

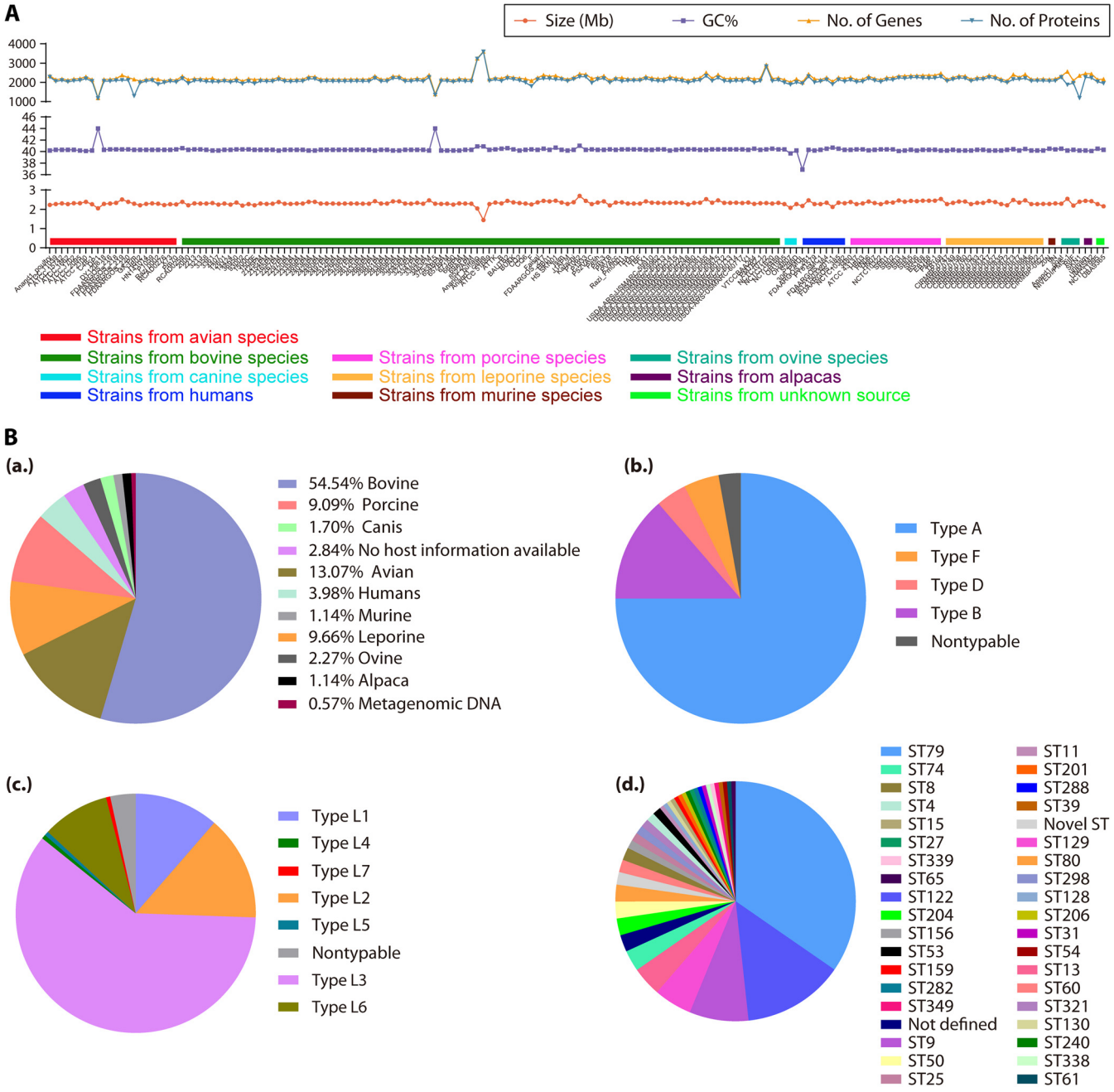

FIG 4 Comparisons among *P. multocida* genomes. Shown are results from comparative analyses of the 176 sequenced *P. multocida* genomes in the NCBI database (as of 31 December 2018). (A) Line charts illustrating whole-genome sizes, average percent G+C content, and numbers of predicted genes and encoded putative proteins. (B) Pie chart showing the host sources (top left), capsular genotypes (top right), LPS genotypes (bottom left), and RIRDC MLST genotypes (bottom right) of the 176 sequenced *P. multocida* genomes in the NCBI database (as of 31 December 2018).

CHARACTERISTICS OF SELECTED COMPLETE *PASTEURELLA MULTOCIDA* GENOMES

The first completed genome of *P. multocida* was that of strain Pm70 (GenBank accession no. AE004439) (23), a serotype F:3 strain isolated in 1976 from the oviduct of a layer chicken with fowl cholera. Although Pm70 has been described as being avirulent for chickens in several studies (16, 77, 78), later sequencing analysis revealed that the isolate used for those animal studies was mutated (J. D. Boyce, M. Harper, and A. D. Cox, personal communication), so the sequenced Pm70 isolate likely is actually a virulent strain (see discussion below). The complete genome sequence of Pm70 is 2,257,487 bp

TABLE 2 General features of *P. multocida* isolates with whole-genome sequences available in the NCBI database[a]

Host species (no. of isolates)	Largest genome size (Mb)	Smallest genome size (Mb)	Avg genome size (Mb)	Average G+C content (%)	Avg no. of genes	Avg no. of proteins
Bovine (96)	2.70	1.44	2.32	40.38	2,226	2,111
Avian (23)	2.51	2.05	2.29	40.47	2,413	2,007
Leporine (17)	2.48	2.32	2.34	40.25	2,250	2,138
Porcine (16)	2.54	2.27	2.39	40.29	2,288	2,169
Human (7)	2.47	2.13	2.29	39.91	2,185	2,070
Ovine (4)	2.54	2.20	2.36	40.3	2,331	1,826
Canine (3)	2.27	2.08	2.21	40.09	2,106	1,960
Murine (2)	2.31	2.28	2.29	40.45	2,164	2,071
Alpaca (2)	2.44	2.42	2.43	40.15	2,453	2,246

[a]As of 31 December 2018.

in length with a GC content of approximately 40.4%, and it contains 2,014 predicted coding regions, 6 rRNA operons, and 57 tRNAs. Analysis of the Pm70 complete genome sequence revealed genes encoding proteins involved in *P. multocida* metabolism, including the oxidative pentose phosphate and Entner-Doudoroff glycolytic pathways, glycolysis, gluconeogenesis, and the Krebs cycle; virulence, including two putative adherence proteins (PfhB1 and PfhB2) with domains homologous to the filamentous hemagglutinin (FhaB) of *Bordetella pertussis*; and iron acquisition. In particular, over 2.5% of the *P. multocida* genome appears to be devoted to genes encoding proteins involved in iron acquisition, suggesting that iron uptake plays an important role in the fitness and virulence of *P. multocida* (23).

The first completed genome of a bovine serogroup A isolate of *P. multocida* was that of strain 36950 (GenBank accession no. CP003022), which was isolated from a case of bovine respiratory tract infection in a Nebraska feedlot (79, 80). Strain 36950 exhibits resistance to most antibiotics commonly used for the control of bovine respiratory diseases, including tetracyclines, chloramphenicol, sulfonamides, spectinomycin, enrofloxacin, florfenicol, tilmicosin, tulathromycin, aminoglycosides, kanamycin, and neomycin (79). Sequence analysis of 36950 uncovered an important mechanism for multidrug resistance in *P. multocida*, namely, an 82.2-kb integrative and conjugative element (ICE) within the chromosome, ICE*Pmu1*, which contains 12 known antibiotic resistance genes (see discussion below).

The first and only completed genome of a toxigenic *P. multocida* strain was that of strain HN06 (GenBank accession no. CP003313), which was isolated from a nasal swab of a swine with progressive atrophic rhinitis in China (81). Genome sequence analysis of HN06 revealed that the *toxA* gene encoding the *P. multocida* toxin (PMT), which is the principal virulence factor for symptoms of progressive atrophic rhinitis, was harbored within an intact prophage area (Fig. 5A). This finding confirmed a previous study that proposed that PMT is encoded by a lysogenic bacteriophage (82). HN06 possesses a 5,360-bp plasmid, pHN06 (GenBank accession no. CP003314), with a G+C content of 47.5%, compared to 40.2% for the chromosome (81). pHN06 contains seven open reading frames (ORFs) that encode putative proteins for plasmid replication, plasmid mobilization, and antibiotic resistance (Fig. 5B), suggesting that pHN06 might confer antibiotic resistance to *P. multocida* HN06.

The first complete genome sequence of a capsular type F strain of *P. multocida* was that of strain HN07 (GenBank accession no. CP007040), which originated from the lung of a swine that died from pneumonia in China (20). Contrary to previous reports that capsular type F strains are generally associated with fowl cholera and are rarely isolated from mammalian hosts (2, 50), the swine strain HN07 was highly pathogenic to swine but avirulent in chickens (20). Analysis of the complete genome sequence of HN07 identified a novel ICE which encodes a putative type IV secretion system (T4SS) and a number of other genomic islands as well as prophages that may contribute to its virulence to swine (20). However, HN07 lacks the putative virulence genes associated with fowl cholera, which might address the question of why HN07 is avirulent in chickens (20).

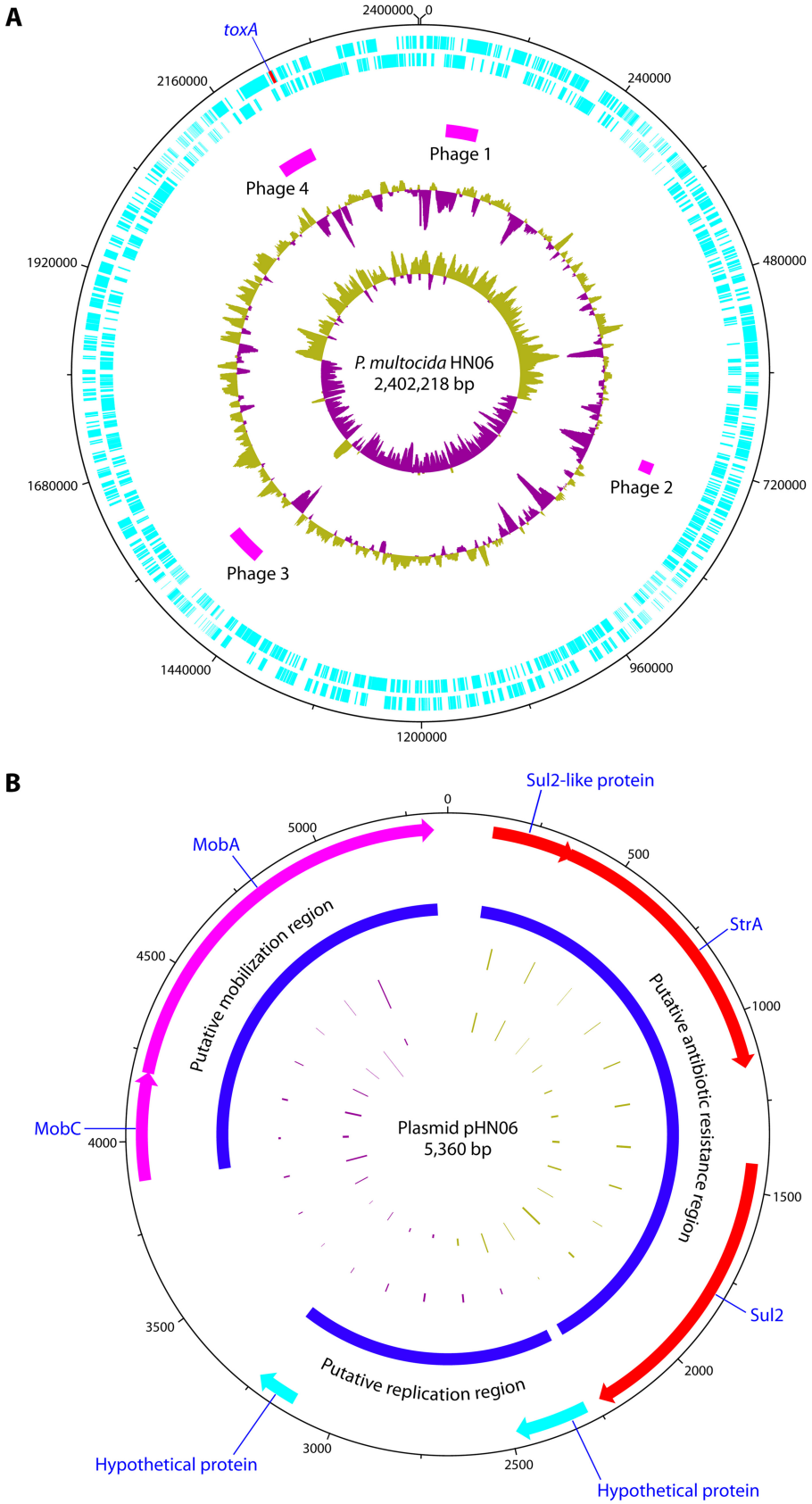

FIG 5 Circle maps of *P. multocida* HN06 genomes. (A) Circle map of the *P. multocida* HN06 chromosomal genome (2,402,218 bp) generated using DNA Plotter (157). From the outside ring to the inside ring, circle

(Continued on next page)

Analysis of the complete genome sequence of a serogroup A isolate of *P. multocida* from bovine in China, strain HB01 (GenBank accession no. CP006976) (17), provided a more detailed metabolic map of *P. multocida*. A number of genes were identified as encoding enzymes involved in carbon utilization from various sources, including mannitol (*mtlADR*), mannose (*manXYZ*), glucose (*pstHI-crr*), sorbitol (*gutM-srlABDE*), sucrose (*scrKABR*), and fructose (*fruABK*), and in responding to nitrates and nitrites, anaerobiosis, and other environmental pressures (e.g., *napFDAGHBC*, *frdABCD*, *arcAB*, *phoBR*, *narPQ*, and *uhpBA*). In particular, two novel genes, *treB* (PMCN01_1241) and *treC* (PMCN01_1239), encoding enzymes involved in trehalose utilization, were identified (17). Other studies have shown that trehalose utilization enhances the virulence and fitness of some pathogenic bacteria, such as *Clostridium difficile*, *Clostridium tyrobutyricum*, and *Klebsiella pneumoniae* (83–85). In addition to these metabolism-related genes, the virulence-associated genes in *P. multocida*, including biosynthesis of capsule, LPS, adherence, sialometabolism, iron regulation, and acquisition proteins and OMPs, were also characterized.

VIRULENCE FACTORS AND VIRULENCE-ASSOCIATED GENES

Many virulence factors contribute to the fitness and pathogenesis of *P. multocida*. Reported virulence factors include capsule, LPS, fimbriae and other adhesins, toxins (PMT), iron-regulated and iron acquisition proteins, sialic acid metabolism, hyaluronidase, enzymes involved in sialic acid metabolism, and a number of OMPs (reviewed in reference 69). *P. multocida* genomes contain a large number of genes encoding proteins involved in the biosynthesis and assembly of these virulence factors (17). This section summarizes and discusses the virulence factor-associated genes carried by the *P. multocida* genome.

Biosynthesis of Capsule

The important role of capsule in the pathogenesis of *P. multocida* has been clearly demonstrated, as the virulence of acapsular mutants constructed from different serogroups was strongly attenuated in mice (86, 87). To date, the capsules of *P. multocida* serogroups A, B, D, and F have been well characterized, and all *P. multocida* capsules are polysaccharides (88). The A-, D-, and F-type capsules are composed of hyaluronic acid, heparin, and chondroitin components, respectively (89). The capsular polysaccharide components of types D and F are closely related but chemically distinct from the type A hyaluronic acid component (88, 89). The type B capsule consists of arabinose, mannose, and galactose components (88). There is still no report about the composition of type E capsule.

Genes involved in the biosynthesis of different *P. multocida* capsule types are located within a single locus (*cap* locus) on the genome (37). Analysis of the genetic organization of the *cap* loci for capsular types A, B, D, and F revealed three separate functional gene clusters: a cluster responsible for capsule biosynthesis, a cluster responsible for phospholipid substitution, and a cluster responsible for capsule export. At least two types of genetic organizations have been determined for the *cap* loci in *P. multocida* (Fig. 6). Type I loci, characteristic of *P. multocida* type A, D, and F strains, consist of 10 genes, *phyBA-hyaEDCB-hexDCBA*, where *phyBA* function in phospholipid substitution, *hyaEDCB* function in capsule biosynthesis, and *hexDCBA* function in capsule export. Type II loci, characteristic of *P. multocida* type B strains, contain 15 genes,

FIG 5 Legend (Continued)
1 shows the DNA base position (base pairs), circle 2 (cyan) shows the protein-coding regions transcribed clockwise, circle 3 (cyan) shows the protein-coding regions transcribed anticlockwise, circle 4 (pink) shows predicted prophages, circle 5 shows the G+C content, and circle 6 shows the GC skew. (B) Circle map of the *P. multocida* HN06 plasmid genome (5,360 bp) generated using DNA Plotter. From the outside ring to the inside ring, circle 1 shows the DNA base position (base pairs); circle 2 (arrows in different colors) shows the protein-coding regions (cyan arrows, putative proteins involved in plasmid replication; purple arrows, putative proteins involved in plasmid mobilization; red arrows, putative proteins involved in antibiotic resistance); circle 3 (blue) shows areas involved in plasmid replication, plasmid mobilization, and antibiotic resistance; circle 4 shows the G+C content; and circle 5 shows the GC skew.

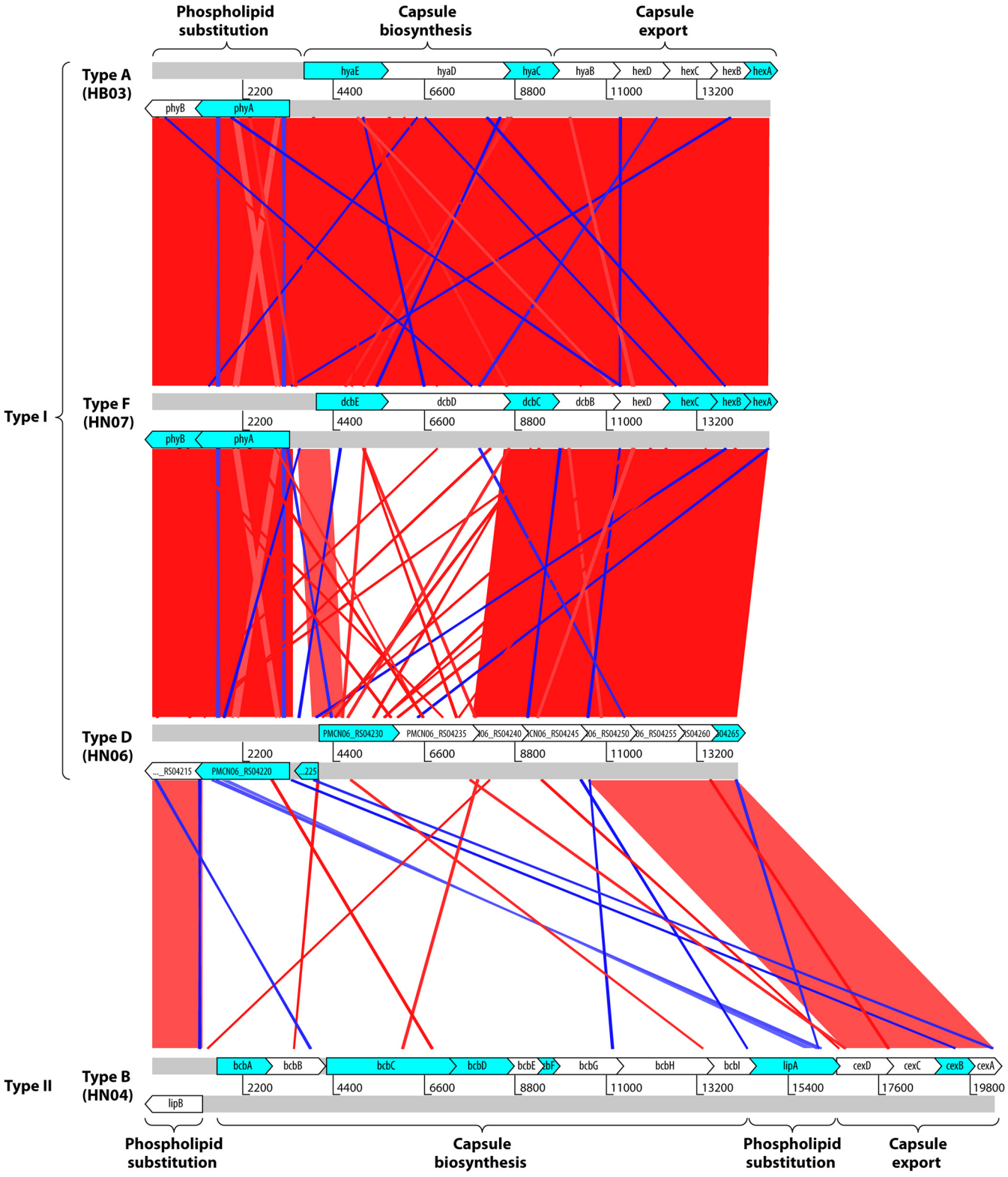

FIG 6 Comparative analysis of capsule-encoding loci among different capsule types visualized by ACT. Shown are Artemis plots (158) of the genomes of type A (HB03), type F (HN07), type D (HN07), and type B (HN04) strains. Arrows (cyan) denote the genes and their direction within the locus. Color-coding denotes the BLASTn identity of these regions between genomes. The red lines (oriented in the same direction) and/or blue lines (oriented in the reverse direction) between the genomes represent DNA-DNA similarities (BLASTn matches) between the two sequences.

lipB-bcbABCDEFGHI-lipA-cexDCBA, where *lipB* and *lipA* function in phospholipid substitution, *bcbABCDEFGHI* function in capsule biosynthesis, and *cexDCBA* function in capsule export. Although a *P. multocida* type E genome is not available, the *cap* locus of capsular type E was found to comprise homologs of *bcbA*, *bcbB*, *bcbC*, *bcbE*, *bcbF*, *bcbG*, and *bcbI*, as those found in a type B *cap* locus (37). Sequence alignments showed that the DNA sequences of the capsule export genes of type A were highly homologous (DNA identity of >95%) to those of types B, D, and F. While the phospholipid substitution genes were highly conserved among types A, D, and F, they all shared less homology (DNA identity of <72%) with those of type B. In contrast, the capsule biosynthesis genes varied among types A, B, D, and F strains, with some genes, such as the glycosyltransferase-encoding genes *hyaD* (in type A), *dcbF* (in type D), and *fcbD* (in type F), displaying higher homology among their respective capsular types but less with others. The type B *cap* locus did not possess any homologs of *hyaD*, *dcbF*, and/or *fcbD*. Instead, the *bcbD* gene in the type B *cap* locus and, similarly, the *ecbJ* gene in the type E *cap* locus represented capsular type-specific target sequences. The capsular polysaccharides of *P. multocida* capsular types A, D, and F, while closely related, are quite distinct from those of type B (88, 89). This unique region could be used to discriminate capsular type B strains from others.

Biosynthesis of Lipopolysaccharide

LPS is also recognized as one of the most important factors associated with the pathogenesis of *P. multocida* (69, 90). The structure of LPS produced by *P. multocida* is composed of a highly hydrophobic lipid A moiety and an inner and outer core oligosaccharide backbone but lacks the typical extended O-antigen structure of LPS found in other Gram-negative bacteria (52). *P. multocida* strains generally produce simultaneously two LPS glycoforms (A and B) that share the same outer core structure but differ in their inner core (53–59). Comparison of representative genomes revealed that the genes required for the biosynthesis and assembly of *P. multocida* lipid A (*lpxA*, *lpxB*, *lpxC*, *lpxD*, *lpxH*, *lpxK*, *lpxL*, *lpxM*, *kdsA*, *kdsB*, and *kdsC*) and the inner core oligosaccharide (*kdkA*, *kdtA*, *gmhA*, *gmhB*, *gmhC*, *hptA*, and *hptB*) are conserved among different *P. multocida* strains, and these genes are located at a number of sites throughout the genome (17, 18, 20). Two of these genes, *hptA* and *hptB*, which encode the heptosyltransferases HptA and HptB, respectively, dictate the production of the inner core of the LPS (52). Thus, the presence of these two genes and whether they are intact in a *P. multocida* genome indicate the types of inner core LPS structures produced by a particular isolate (17, 18, 21).

Unlike the inner core assembly genes, genes required for the biosynthesis of the LPS outer core are located within a single locus on the genome, between the two conserved genes *fpg* and *priA* (17, 52, 90). This gene cluster shares different levels of DNA homology among strain types and determines the eight unique LPS outer core biosynthesis loci (L1 through L8) (Fig. 1). Among these loci, the L6 locus shares high sequence homology with the L3 locus, while the L4 locus shows some sequence homology to the L8 locus, but otherwise, there is limited sequence similarity among the loci.

Biosynthesis of Fimbriae and Other Adhesins

P. multocida genomes have several genes, *ptfA*, *comE1*, and *pfhB*, that code for putative proteins with similarity to fimbriae and other adhesins found in other bacteria (91). The *ptfA* gene encodes the main subunit of type 4 fimbriae (pili) (69), which is a characteristic trait found in *P. multocida* isolates with different serotypes/genotypes and from different hosts (19, 36, 51). For instance, the gene *ptfA* (PM0084) from Pm70 is also present (100% DNA identity) in the genomes of *P. multocida* strains P1059 (type A; avian isolate), X73 (type A; avian isolate), 36950 (type A; bovine isolate), HN06 (type D; porcine isolate), and 3480 (type A; porcine isolate) (16–19, 23). *comE1* (Pm70-PM1665) encoding a putative adhesin is present (≥99% DNA identity) in the genomes of strains Pm70, P1059, X73, 36950, HN06, and 3480 (16). The gene *pfhB* encodes a filamentous

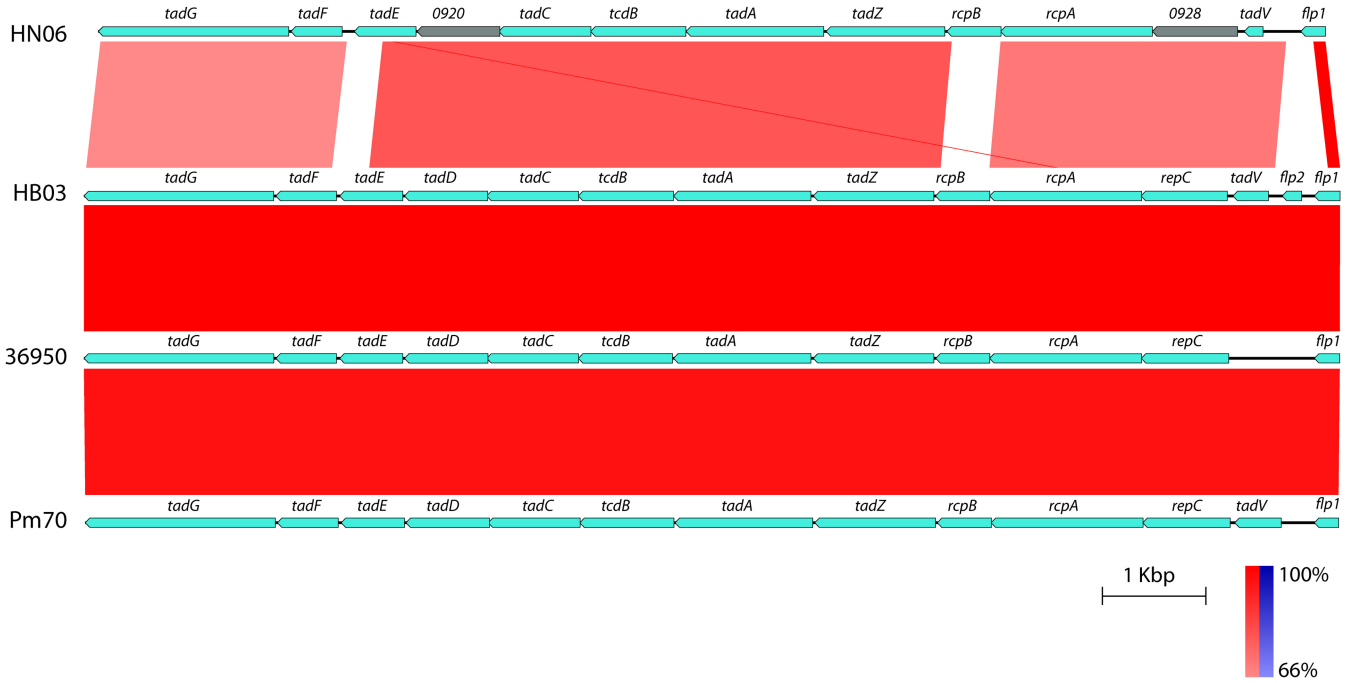

FIG 7 Genetic organization and colinearity of the *tad* locus among selected *Pasteurella multocida* strains. Shown are synteny plots for the *tad* loci of *P. multocida* strains HN06, HB03, PM70, and 36950, visualized by using EasyFig 2.2.3 (155). Cyan arrows denote the genes and their direction within the loci; gray arrows denote genes with frameshifts.

hemagglutinin (FhaB) protein, with similarity to the FhaB protein of *Bordetella*, which is known to play a critical role in adherence of the *Bordetella* bacterium to the respiratory epithelium (92). The Pm70 genome possesses two copies of *pfhB* (23): *pfhB1* (PM0057) is present (≥98% DNA identity) in strains P1059, X73, and 3480 but not in 36950 or HN06, while *pfhB2* (PM0059) is present (≥90% DNA identity) in strains P1059, X73, and 36950 but not in HN06 or 3480 (16). Some *P. multocida* isolates contain additional FhaB-encoding genes. Sequence analysis of the P1059 genome identified two additional *pfhB* genes, besides *pfhB1* and *pfhB2* (16): the first one, *pfhB3*, is present (≥96% DNA identity) in strains X73 and 36950 but not in Pm70, HN6, or 3480, while the second one, *pfhB4*, is present (≥93% DNA identity) in strains HN06 and 3480 but not in Pm70, X73, or 36950 (16).

The *tad* locus is required for the assembly of low-molecular-weight fimbrial adhesin protein and is essential for biofilm formation, colonization, and pathogenesis in many members of the family *Pasteurellaceae* (93). In general, the *tad* locus is composed of 14 genes (*flp1-flp2-tadV-rcpCAB-tadZABCDEFG*). For example, the *tad* locus in strain HB03 (GenBank accession no. CP003328) is intact and includes all 14 genes. However, some strains of *P. multocida* do not possess an intact 14-gene *tad* locus. While the *tad* locus in strain HB03 is highly homologous (>95% DNA identity) to those found in strains 36950, HB01, and Pm70 (17), the *flp2* gene is missing in strains HB01, HN06, 36950, and Pm70 (17); the *rcpC* gene is missing in strain 36950; and a frameshift mutation is found within the *rcpC* (0928) and *tadD* (0920) genes in strain HN06 (Fig. 7). The *tad* locus in strain HN06 also shares less homology (<66% DNA identity) with those of strains HB03, 36950, HB01, and Pm70. The proteins (Flp1-TadV-RcpCAB-TadZABCDEFG) encoded by the genes in the *flp* operon of strain HN06 have 73%, 66%, 58%, 69%, 42%, 75%, 93%, 78%, 79%, 62%, 54%, 62%, and 60% amino acid similarities to those encoded by the *flp* operon in strain HB03, respectively.

The G-Protein-Deamidating Toxin

The 146-kDa, single-chain, G-protein-deamidating toxin of *P. multocida* (PMT), the etiological agent responsible for progressive atrophic rhinitis and dermonecrosis (2, 3,

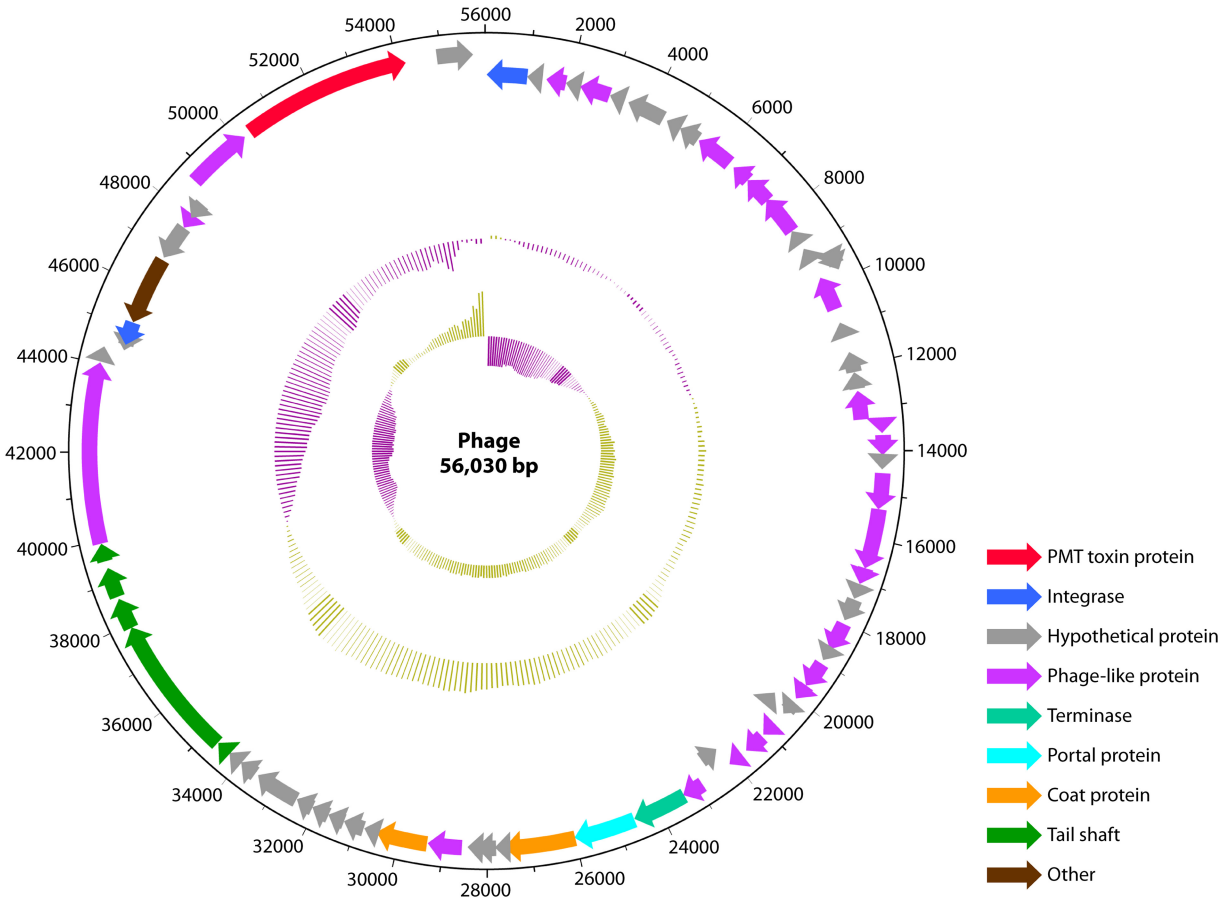

FIG 8 Genome of a toxigenic *Pasteurella multocida* strain HN06 phage carrying the PMT-encoding gene. Shown is a circular map of the *P. multocida* strain HN06 carrying an intact *toxA* gene-containing prophage, generated by using DNA Plotter (157). From the outside to the inside rings, circle 1 shows the DNA base position (base pairs), circle 2 (arrows in different colors) shows the protein-coding regions, circle 3 shows the G+C content, and circle 4 shows the GC skew.

69), is encoded by the 3,858-bp *toxA* gene, which is located on a predicted 56,030-bp intact prophage adjacent to a tRNA gene ($tRNA^{Trp}$) in the toxigenic *P. multocida* strain HN06 isolated from a case of progressive atrophic rhinitis in swine (81) (Fig. 5A). Sequence analysis of DNA flanking the *toxA* gene revealed the presence of upstream genes encoding proteins involved in phage structure and function (Fig. 8). This lysogenic prophage was reported to be inducible upon treatment with mitomycin C, with the resulting phage having morphological characteristics similar to those of phages belonging to the *Siphoviridae* family (82). To date, knowledge about the regulation and export of PMT remains unknown, but it has been speculated that a bacterial SOS response induced by environmental factors encountered during host infection might lead to induction of the phage lytic cycle, thereby causing bacterial cell lysis and toxin release (82).

Iron Regulation and Acquisition

Iron acquisition has been proposed as a key biological process for *P. multocida* survival and pathogenesis in the host (3). This is supported by the fact that over 2.5% of the total genes (53 genes) found in the Pm70 genome are predicted to encode proteins involved in iron uptake or acquisition (23). Likewise, more than 2.1% of the total genes (46 genes) in the HB01 genome are predicted to encode proteins devoted to iron uptake or acquisition (17). Predicted genes or operons shown to have roles in iron metabolism include the *tonB-exbB-exbD* operon that encodes proteins forming the TonB-dependent transporter (TBDT) required for energizing the iron transport process

(94); the *fecABCDE* operon that encodes proteins involved in the synthesis of the TBDT system (17); the *tbpA* gene that encodes transferrin-binding protein A important for iron uptake in *Pasteurellaceae* (69); the *afuCBA* operon that encodes the Afu iron transport system, similar to that found in *Actinobacillus pleuropneumoniae* (95); the *yefABCD* and *fbpABC* operons that encode proteins homologous to the periplasmic iron transport systems YefABCD and FbpABC found in *Mannheimia haemolytica* (96); the *ccmABCDEF* operon that encodes proteins involved in posttranslational attachment of heme, found in *Escherichia coli* (97); the *hgbA* gene that encodes a hemoglobin-binding protein similar to that found in *A. pleuropneumoniae* (98); the *fur* gene that encodes a ferric uptake regulator, Fur, similar to that found in *A. pleuropneumoniae* (99); the *hmuVUT* operon that encodes proteins homologous to a family of ABC transporters (HemTUV and ShuTUV) involved in transporting free heme into the cytoplasm, similar to that found in *Haemophilus parasuis* (100); the *fdx-hscAB-iscAUSR* operon that encodes the Fe-S assembly machinery, similar to that found in *Xenorhabdus nematophilus* (101); and others. Most of these iron acquisition systems are highly conserved among *P. multocida* strains that produce similar types of capsular polysaccharide (types A, D, and F) (17). It is noteworthy that iron acquisition is often considered an important restricting factor for host specificity (3). A noteworthy protein is TbpA, the only transferrin receptor identified in *P. multocida*, that is encoded by *tbpA*, which is present in only some isolates from bovine and ovine species (36, 69).

Sialic Acid Metabolism

Putative genes involved in the sialometabolism of *P. multocida* include *nanB*, *nanH*, *neuS2*, *neuA*, the *nan* operon (*nanRATEK-yhcH*), and the *siaPT-nanM* cluster (17). Among these genes, *nanB* and *nanH* encode the two key sialidases NanB and NanH that help with sialic acid uptake (102), *neuS2* encodes a protein homologous to a sialyltransferase found in *Haemophilus influenzae* (103), and *neuA* encodes a CMP-sialic acid synthetase similar to that which activates the precursor scavenging pathway involved in the uptake of environmental sialic acid in *E. coli* (104). The members of the *nan* operon (*nanRATEK-yhcH*) encode structural (NanATEK) and regulatory (NanR) proteins similar to those found in *H. influenzae* that are involved in sialic acid catabolism (105), while the *siaPT-nanM* cluster, which lies downstream of the *nan* operon on the opposite strand, encodes proteins that form the TRAP system required for sialic acid uptake (103).

Outer Membrane Proteins

Bacterial OMPs play key roles in nutrient acquisition, molecule and ion transport, and bacterium-host interactions (91). According to their functional characteristics, *P. multocida* OMPs are mainly categorized into structural proteins, transport proteins, binding proteins, adhesins, protein assembly machinery, and membrane-associated enzymes (91). Analysis of the Pm70 genome identified 72 genes (approximately 3.57% of the total genes) encoding putative proteins that functioned as OMPs (91). Among those OMPs identified, structural proteins (PM0786 and PM0966), transporters (PM0527 and PM1980), binding lipoproteins (PM1730 and PM1578), and membrane-associated enzymes (PM1426, PM1000, and PM1992) are relatively conserved among *P. multocida* isolates associated with a range of clinical manifestations in different hosts, while those OMPs with predicted functions, such as hemoglobin receptors (PM0040, PM0236, PM0300, PM0337, PM0576, PM0592, PM1081, PM1282, PM1622, and PM1188) or adhesins (PM0056, PM0057, PM0058, PM0059, PM0084, PM1665, PM0844, PM0846, PM0852, PM0853, and PM0855), are relatively heterogeneous (Fig. 9).

COMPARATIVE GENOME ANALYSIS OF *P. MULTOCIDA* ISOLATES WITH DIFFERENT CHARACTERISTICS

P. multocida is a heterogeneous species, and strains of *P. multocida* from different clinical presentations can be assigned into different serogroups or genotypes (A, B, D, E, and F) (24, 37) and/or subspecies (106, 107). They are also classified into toxigenic and nontoxigenic strains based on whether they carry the toxin-encoding gene *toxA* and

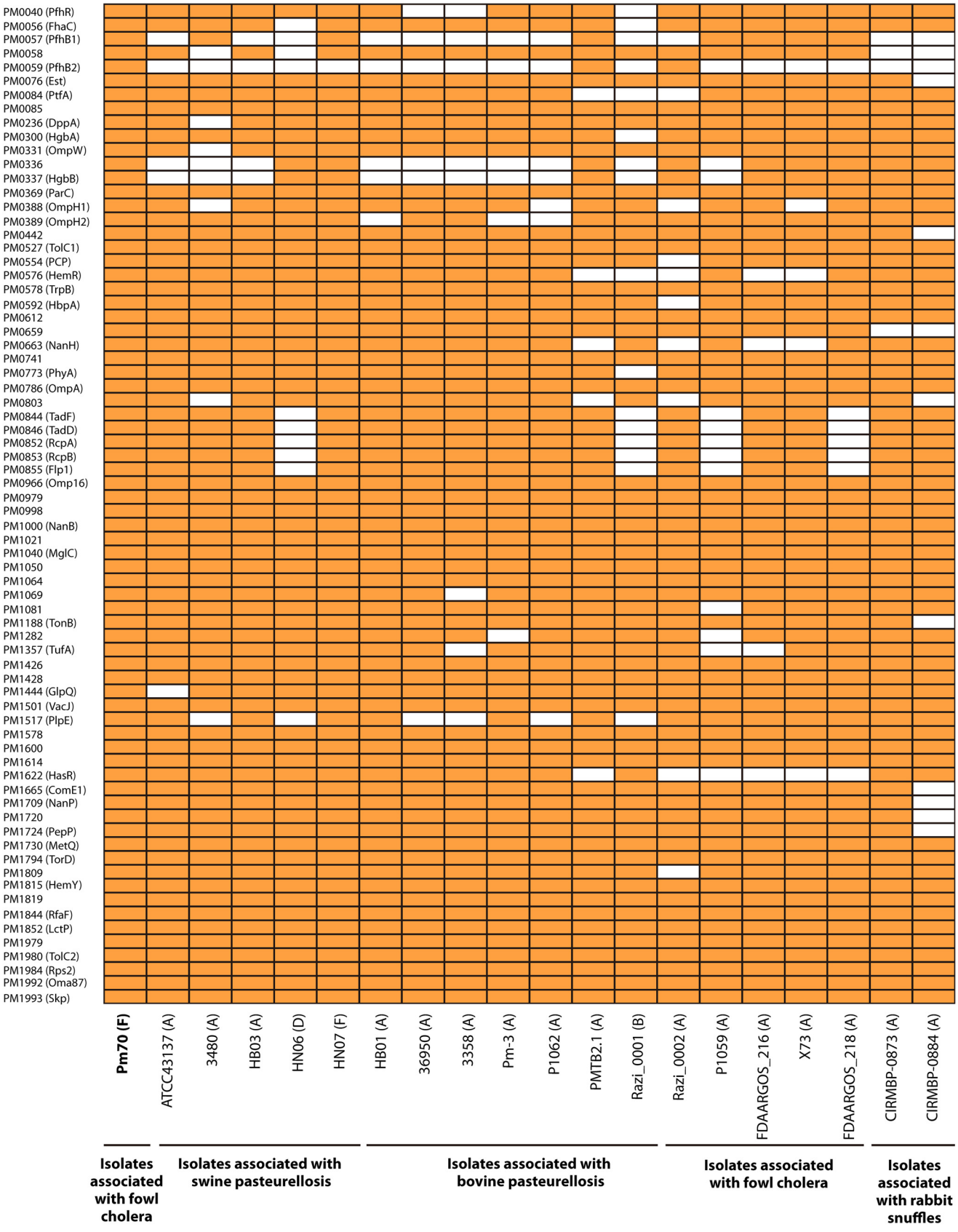

FIG 9 Heat map showing the presence of putative OMP-encoding genes among *P. multocida* isolates associated with different diseases in different hosts. Conserved genes (shown in orange) were determined using BLASTn with an identity of ≥80% plus alignment coverage of ≥80% and an E value of 1e−6 as the cutoff.

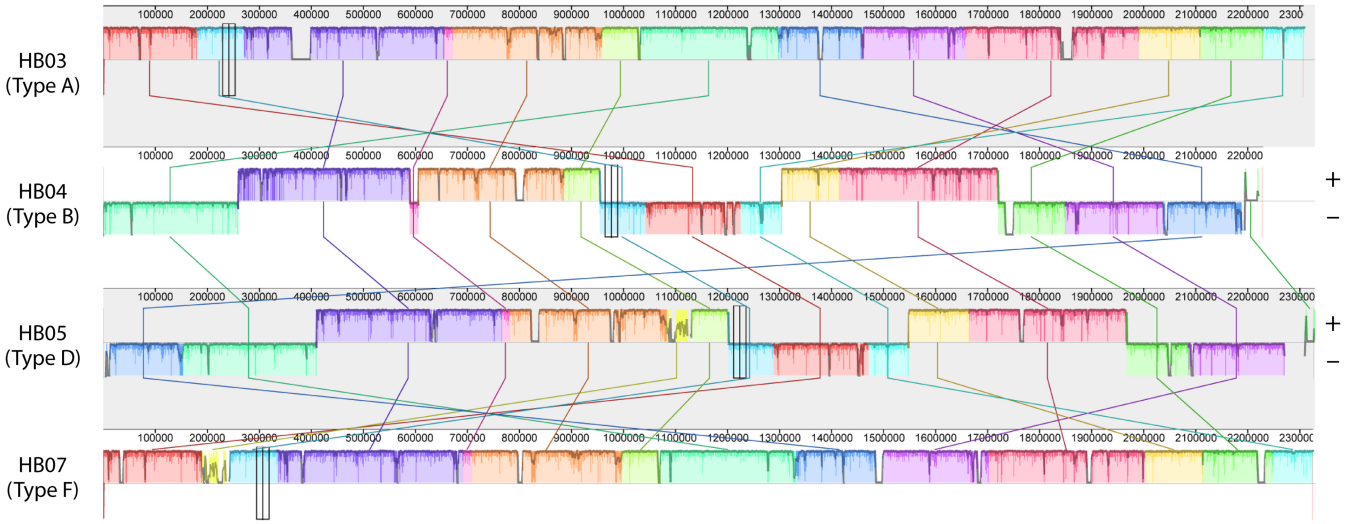

FIG 10 Colinearity of the representative genome sequences of selected isolates of different serogroups/genotypes from swine. Shown are genome alignments for *P. multocida* strains HB03 (type A), HN03 (type B), HN05 (type D), and HN07 (type F), generated using the progressiveMauve program (159). Rectangles of similar colors show colinear blocks of genes. In the HN04 and HN05 panels, the top colored blocks labeled "+" indicate genes oriented in the same direction as in the HB03 genome, while blocks labeled "−" indicate genes oriented in the reverse direction. Areas of low identity within the colinear blocks are shown by a reduced height of shading.

produce the toxin (PMT), even though both types are capable of causing infection (2, 3). While there is still no genome sequence publicly available for *P. multocida* serogroup E, the genome sequences of a number of different isolates of *P. multocida* serogroups A, B, D, and F with various characteristics have become available. In this section, comparative genomics among the *P. multocida* isolates are discussed.

Different Genotypes Associated with Swine Pasteurellosis

Comparative genomics of nontoxigenic *P. multocida* serogroups/genotypes A (strain HB03), B (strain HN04; GenBank accession no. PPVE00000000), D (strain HN05; GenBank accession no. PPVE00000000), and F (strain HN07; GenBank accession no. CP007040) from swine identified a number of unique regions for each of the genomes (Fig. 10). The first noteworthy HB03-specific region harbored by the genome (361.3 to 398.7 kb) (Fig. 10) is a prophage with similarity to a Mu-like bacteriophage. Another HB03-specific region (1,374.4 to 1,382.8 kb) (Fig. 10) appears to be involved in trehalose utilization and consists of genes encoding putative proteins with homology to an NADPH-dependent flavin mononucleotide (FMN) reductase (PMCN03_1257), a trehalose-6-phosphate hydrolase (PMCN03_1260), trehalose-specific IIC component TreB (PMCN03_1262), and the helix-turn-helix (HTH)-type transcriptional regulator TreR (PMCN03_1263). The third HB03-specific region (1,844.5 to 1,862.4 kb) (Fig. 10) is a putative acquired genetic element containing genes associated with tetracycline resistance. This region contains several *tet* genes encoding the tetracycline ribosomal protection proteins that confer resistance to tetracyclines, including tetracycline resistance protein D (PMCN03_1714), a *tet*(B) operon (PMCN03_1715), and a tetracycline resistance class B protein (PMCN03_1716). This region also contains genes encoding proteins involved in DNA conjugation, transduction, and/or transformation, including a transposase (PMCN03_1706), two IS*10* transposases (PMCN03_1713 and PMCN03_1717), a transposition helper protein (PMCN03_1707), modification methylase AccI (PMCN03_1711), and helicase IV (PMCN03_1719). Since *tet* genes are disseminated through bacterial populations on mobile genetic elements (108), finding genes encoding Tet-related proteins in the same regions as proteins involved in conjugation, transduction, and transformation strongly suggests that genetic mobility might have contributed to the acquisition of this unique region in the HB03 genome and that this region might play a role in the horizontal spread of tetracycline resistance.

The genome of the serogroup B strain HN04 contains three unique regions that are missing in strains HB03, HN05, and HN07. The first region (791.0 to 811.5 kb) (Fig. 10) contains genes encoding proteins associated with capsular polysaccharide biosynthesis, including a capsular polysaccharide export system protein, KpsS (PMCN04_0732); a UDP-*N*-acetylglucosamine 2-epimerase (PMCN04_0733); a UDP-*N*-acetyl-D-mannosamine dehydrogenase (PMCN04_0734); two capsular polysaccharide biosynthesis proteins (PMCN04_0738 and PMCN04_0739); a capsular polysaccharide biosynthesis and export periplasmic protein, WcbC (PMCN04_0743); a capsular polysaccharide export system inner membrane protein, KpsE (PMCN04_0744); a capsular polysaccharide ABC transporter and permease protein, KpsM (PMCN04_0745); and a capsular polysaccharide ABC transporter and ATP-binding protein, KpsT (PMCN04_0746). The second unique region (1,733.4 to 1,749.8 kb) (Fig. 10) is predicted to be a prophage with similarity to the *Salmonella* lambda phage 118970_sal3 (GenBank accession no. NC_031940). The third region (2,187.6 to 2,218.2 kb) (Fig. 10) is also predicted to be a Mu-like bacteriophage.

The genome of the serogroup D strain HN05 contains five specific regions that are missing in strains HB03, HN04, and HN07. The first region (820.7 to 837.5 kb) (Fig. 10) is a prophage with similarity to the *Enterobacteria* lambda bacteriophage VT2-Sakai (GenBank accession no. NC_000902). The second region (974.3 to 981.4 kb) (Fig. 10) contains genes encoding proteins (PMCN04_0907 and PMCN04_0906) with similarity to the HigBA toxin-antitoxin system. The third region (1,119.7 to 1,131.1 kb) (Fig. 10) contains genes encoding proteins involved in DNA replication, including a DNA topoisomerase III (PMCN05_1054) and a single-stranded DNA-binding protein (PMCN05_1057), and in chromosome partitioning, including an ATPase (PMCN05_1064) and a DNA helicase, DnaB (PMCN05_1063). The fourth region (1,761.0 to 1,769.5 kb) (Fig. 10) contains a cluster of genes encoding proteins comprising a type I restriction-modification system, including restriction subunit R (PMCN05_1674), an anticodon nuclease (PMCN05_1675), specificity subunit S (PMCN05_1676), a putative DNA-binding protein (PMCN05_1677), and DNA methyltransferase subunit M (PMCN05_1678). The restriction-modification system presumably protects the host bacterium from invasion by foreign DNA through global methylation by the putative methyltransferase activity and digestion of the invaded DNA by the putative restriction endonuclease activity (109). In an early study, a PstI methylase-based restriction-modification system that was discovered in the toxigenic *P. multocida* strain LFB3 prevented conjugal transfer of several naturally occurring antibiotic resistance plasmids isolated from *P. multocida* type D strains (110). The fifth region (2,266.3 to 2,328.9 kb) (Fig. 10) is also predicted to be a *Mannheimia* lambda-like bacteriophage, vB_MhS_1152AP2 (GenBank accession no. NC_028956) (111).

The genome of the serogroup F strain HN07 possesses seven unique regions that are missing in strains HB03, HN04, and HN05. The first region (31.1 to 37.6 kb) (Fig. 10) contains genes encoding proteins comprising a type II-C CRISPR-Cas system, including CRISPR-associated proteins Cas9 (PMCN07_0036), Cas1 (PMCN07_0037), and Cas2 (PMCN07_0038). This type II-C CRISPR-Cas system presumably establishes genetic immunity against the entry of mobile genetic elements (112). The second unique region (188.7 to 243.7 kb) (Fig. 10) has striking homology to an ICE designated ICE*pmcn07* (20). This ICE carries genes encoding a putative T4SS (20), which may serve as the DNA conjugation system for horizontal transfer of ICE*pmcn07* (113) (see discussion below). The third region (796.7 to 805.8 kb) (Fig. 10) contains genes encoding proteins comprising a putative type III restriction-modification system, including a helicase domain protein (PMCN07_0715), a methyl-accepting chemotaxis protein (PMCN07_0716), adenine-specific DNA methylase Mod (PMCN07_0717), and a type III restriction enzyme Res subunit (PMCN07_0718). Type III restriction-modification systems have important roles in the biology of bacterial pathogens, including DNA restriction activity and gene regulation (114). The fourth unique region (1,483.8 to 1,497.5 kb) (Fig. 10) contains genes encoding proteins involved in DNA conjugation and recombination, including a phage integrase family site-specific recombinase (PMCN07_1309), a putative integrase (PMCN07_1310), a transposase (PMCN07_1312), and a second putative transposase

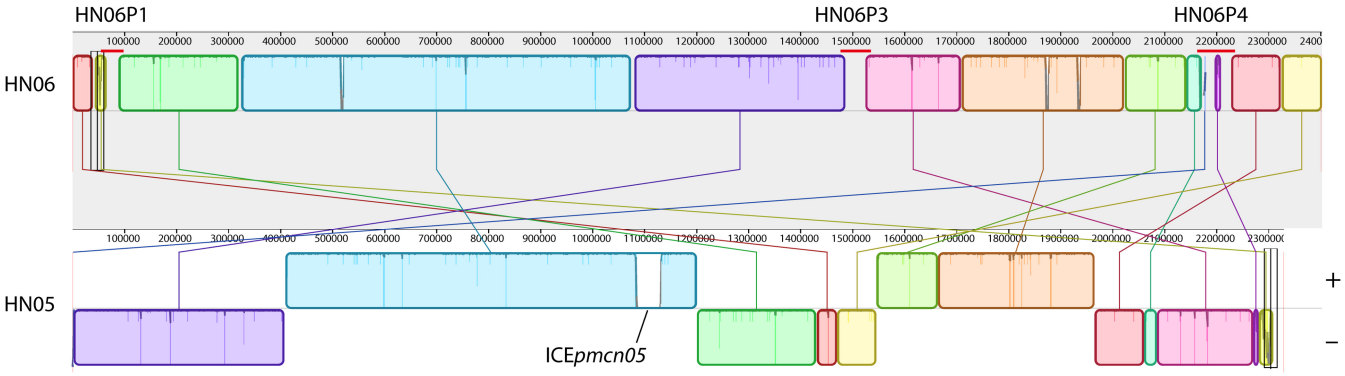

FIG 11 Colinearity of toxigenic versus nontoxigenic *Pasteurella multocida* strains. Shown are genome sequence alignments of toxigenic *P. multocida* strain HN06 (top) and nontoxigenic strain HN05 (bottom), generated using the progressiveMauve program (159). Rectangles of similar colors show colinear blocks of genes. In the HN05 panel, the top colored blocks labeled "+" indicate genes oriented in the same direction as in the HN06 genome, while blocks labeled "−" indicate genes oriented in the opposite direction. Areas of low identity within the colinear blocks are shown by reduced shading.

(PMCN07_1324). In addition, this region also encodes several proteins with putative roles in enhancing bacterial fitness. For example, the presence of an arsenate reductase (PMCN07_1315) and two ACR3 arsenic resistance proteins (PMCN07_1316 and PMCN07_1319) confers resistance to arsenic (115), an alcohol dehydrogenase (PMCN07_1320) might play a role in ethanol tolerance (116), and a flavodoxin protein (PMCN07_1323) might have a role similar to that found in a variety of photosynthetic and nonphotosynthetic reactions in bacteria (117). The fifth unique region (1,679.8 to 1,687.4 kb) (Fig. 10) also contains genes encoding proteins beneficial for bacterial fitness. The oxidoreductases (PMCN07_1494 and PMCN07_1500) might have a role in the detoxification of xenobiotic organic compounds by promoting the transfer of electrons from a reduced organic substrate to another chemical compound (118). The maltose/maltodextrin ABC transporter permease protein MalF (PMCN07_1498) participates in the uptake and metabolism of glucose polymers (maltodextrins), which is a class of nutrients utilized by many bacteria in both mammalian hosts and the environment (119). The putative glycerol-3-phosphate (G3P) transporter membrane protein (PMCN07_1499) might help to transport G3P into the cytoplasm and so may play a major role in glycolysis and phospholipid biosynthesis as in other bacteria (120). The sixth unique region (1,890.2 to 1,899.3 kb) (Fig. 10) contains genes encoding proteins conferring resistance to phage DNA, including a type I restriction-modification system M subunit (PMCN07_1695), an HsdA protein (PMCN07_1696), an abortive infection bacteriophage resistance-like protein (PMCN07_1698), and a type I site-specific DNase belonging to the HsdR family (PMCN07_1699). The seventh unique region (2,218.4 to 2,232.6 kb) (Fig. 10) appears to be a *Salmonella* lambda-like bacteriophage, 118970_sal3 (GenBank accession no. NC_031940).

Toxigenic and Nontoxigenic Isolates from Swine

Some *P. multocida* isolates from swine (45, 121, 122) and ovine (123, 124) species express the toxin PMT, which is a 1,285-amino-acid protein with G-protein deamidase activity that causes various mitogenic and cytopathic effects on host cells (125). Toxigenic isolates are mainly of capsular type D (genotype D:L6:RIRDC ST50) (45, 51), but there are some reports of toxigenic type A strains (66). Currently, there is only one whole-genome sequence that is publicly available for a toxigenic isolate, strain HN06 (81). Sequence comparison revealed that the genomes of strain HN06 and the nontoxigenic capsular type D strain HN05 (genotype D:L6:RIRDC ST50) are highly colinear and contain 12 large, locally colinear blocks (Fig. 11). The genome of the toxigenic strain HN06 contains three large regions that are not found in the genome of strain HN05 (65.9 to 89.3 kb, 1,485.0 to 1,524.4 kb, and 2,178.0 to 2,231.7 kb) (Fig. 11). Interestingly, these regions are located within the three intact prophage regions of strain HN06. However, parts of these three prophages are also present in the genomes

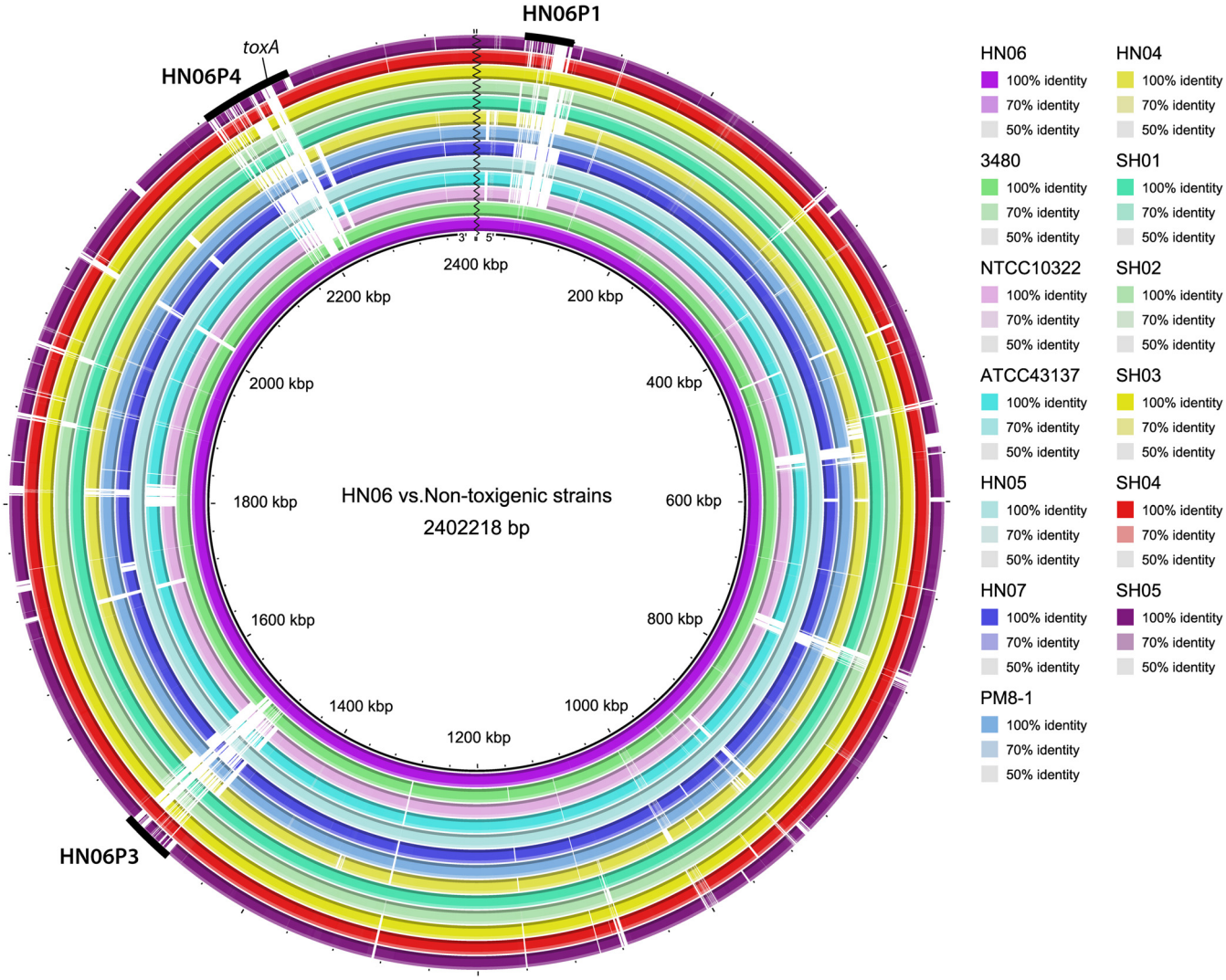

FIG 12 Comparative genomics of toxigenic and selected nontoxigenic strains of *Pasteurella multocida* from swine. Shown are circular maps of genome sequences of *P. multocida* isolates from swine, generated using the BRIG package (160). Alignments include a toxigenic strain (HN06) and nontoxigenic strains (3480, NTCC10322, ATCC 43137, HN05, HN07, PM8-1, HN04, SH01, SH02, SH03, SH04, and SH05). DNA identities between each of the genome sequences are shown. The functions of the regions displaying low identities between different genome sequences are marked as bars at the outermost circle. Most genes within the areas marked by the black bar encode hypothetical proteins.

of many other nontoxigenic isolates (Fig. 12). Among these three intact prophages, the third one located within the 2,178.0- to 2,231.7-kb region in the genome of strain HN06 harbors the PMT-encoding gene *toxA* (Fig. 12). Interestingly, a 6.7-kbp segment of this sequence that lacks the *toxA* gene is also present in the genome of a nontoxigenic isolate (strain 3480) from a pneumonia case (Fig. 12) (3). The genome of the nontoxigenic strain HN05 also contains a 47.9-kb region not found in strain HN06 (1,083.1 to 1,131.1 kb) (Fig. 11). Further analysis predicted that this region is a putative ICE, designated ICE*pmcn05* (see discussion below).

Different Subspecies Associated with Fowl Cholera

P. multocida subsp. *multocida*, *gallicida*, and *septica* are all capable of inducing fowl cholera (reviewed in references 126–130). Comparison of representative genomes of *P. multocida* subsp. *multocida* (C48-1; GenBank accession no. LODS00000000), *P. multocida* subsp. *gallicida* (P1059; GenBank accession no. AMBQ01000000), and *P. multocida* subsp. *septica* (HB02; GenBank accession no. LYOX00000000) revealed high colinearity with 19 large locally colinear blocks (Fig. 13). While strain HB02 does not possess any

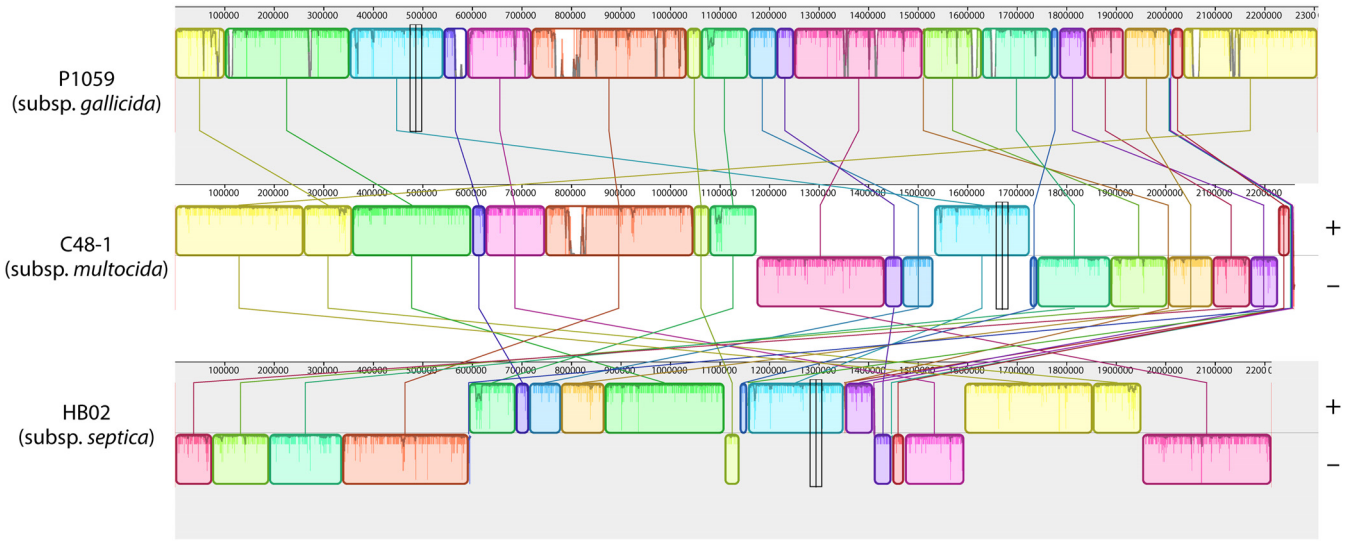

FIG 13 Colinearity of representative genomes of *Pasteurella multocida* subspecies. Shown are genome alignments of *P. multocida* subsp. *multocida* (C48-1), *P. multocida* subsp. *gallicida* (P1059), and *P. multocida* subsp. *septica* (HB02), using the progressiveMauve program (159). Rectangles of similar colors show colinear blocks of genes. In the panels for strains C48-1 and HB02, the top colored blocks labeled "+" indicate genes oriented in the same direction as in the P1059 genome, while blocks labeled "−" indicate genes oriented in the opposite direction. Areas of low identity within the colinear blocks are shown by reduced shading.

large unique regions within its genome, strain C48-1 contains one large unique region (772.0 to 843.0 kb) (Fig. 13) predicted to be a putative prophage with similarity to a *Mannheimia* lambda-like bacteriophage, vB_MhS_535AP2 (GenBank accession no. NC_028853) (111). In contrast, the genome of strain P1059 possesses many unique regions that are not found in the genomes of strains C48-1 and HB02.

One unique region present in the genome of strain P1059 (107.9 to 115.7 kb) (Fig. 13) but absent from the genomes of strains C48-1 and HB02 contains six genes encoding proteins (P1059_00080 to P1059_00085) predicted to be involved in the transport and modification of citrate and the subsequent conversion of citrate to oxaloacetate via citrate lyase (16). A second region (773.1 to 804.3 kb) (Fig. 13) is a putative prophage with homology to a *Mannheimia* lambda-like bacteriophage, vB_MhS_1152AP2 (GenBank accession no. NC_028956) (111). A third region (1,608.2 to 1,618.9 kb) (Fig. 13) contains 10 genes encoding proteins involved in the transport and utilization of L-fucose (P1059_01496 to P1059_01503), an important component of host mucin, which might confer a fitness advantage for *P. multocida* strains in low-nutrient environments (16). The last unique region (1,647.4 to 1,656.9 kb) (Fig. 13) contains genes encoding proteins involved in metabolism and transport of xylose (P1059_01535 to P1059_01541) (16).

Comparative Genome Analysis of Isolates with Different Virulence Capabilities

Comparative genomic analysis of *P. multocida* isolates with different virulence capabilities is a powerful tool to identify putative pathogenesis-associated genes (131). In principle, this is possible only for isolates from the same host, as the pathogenicity of *P. multocida* displays host predilection. For example, nonavian isolates generally do not cause fowl cholera in birds (2). To date, relatively few studies have performed comparative genomic analysis of nonavian strains of *P. multocida* to determine the virulence capacities of the isolates. This may be due in part to the inconvenience and expense associated with the use of large animals such as porcine, ovine, and bovine species.

Some studies have explored the use of murine models instead of natural hosts for pathogenicity determination. For example, a recent comparative genomics study using a murine infection model with two different bovine isolates of *P. multocida* identified several virulence-associated genes present in the genome of the highly virulent

isolate PmCQ2 (GenBank accession no. NZ_LIUN00000000) that were absent in the genome of the isolate PmCQ6 with low virulence (GenBank accession no. NZ_LIUO01000000) (132). These genes included those encoding a putative UDP-3-*O*-acylglucosamine *N*-acyltransferase (putative LPS-modifying enzyme); a putative noncanonical purine nucleoside triphosphate (NTP) pyrophosphatase (purine metabolism); a putative iron-binding protein, FbpA; a putative periplasmic serine protease Do/HhoA-like protein; a putative SrfC-like T3SS effector protein; and elongation factor Tu.

An early report of comparative genomics analysis of a putative avirulent avian strain, Pm70 (GenBank accession no. AE004439), with two virulent strains, P1059 (GenBank accession no. AMBQ01000000) and X73 (GenBank accession no. AMBP01000000), identified several candidate genes involved in fitness and pathogenicity (16). However, as mentioned above, it was later revealed that the so-called "avirulent" strain Pm70 used in that study should actually be considered a virulent strain (J. D. Boyce, M. Harper, and A. D. Cox, personal communication). This discrepancy resulted from the fact that despite the initial genome report stating that the isolate was from a case of avian fowl cholera (23), a later report indicated that the Pm70 strain did not infect chickens, indicating that the Pm70 strain was avirulent (77). Therefore, it was concluded in a subsequent comparative genomics study (133), and in other comparative studies (16, 77, 78), that many of the putative virulence genes identified in the genomes of strains P1059 and X73, but missing in the genome of strain Pm70, were not important for pathogenesis (16). However, it was later discovered from sequencing of the LPS biosynthesis loci of various strains that the Pm70 isolate used for the previous animal studies had a point mutation in the gene for GctC (deletion of A at position 173 in *gctC*) that was not present in the original sequenced strain. This point mutation resulted in a truncated LPS that rendered their strain avirulent in chickens (J. D. Boyce, M. Harper, and A. D. Cox, personal communication). Nevertheless, the comparative genome analysis studies (16, 133) provided some new insights regarding the molecular basis for the fitness of these virulent isolates of *P. multocida*. For example, the genomes of both strains P1059 and X71 contain a region comprised of 10 genes (P1059, 01496 to 01503; X73, 01400 to 01407) that encode predicted proteins with similarity to those involved in L-fucose transport and utilization. Although this region is not found in the genome of strain Pm70 or in the genomes of other reported virulent avian strains, such as strains HB02 (GenBank accession no. LYOX00000000) and GX-Pm (GenBank accession no. JZXO00000000) (18, 131), it may confer a fitness advantage for some avian strains in the respiratory tract, enabling them to utilize the fucose component of host mucin as a nutrient source (16).

Comparative Genome Analysis of *Pasteurella multocida* Isolates from Different Hosts

As noted above, *P. multocida* isolates are capable of causing a wide spectrum of diseases in a wide range of host species. A number of articles have documented that *P. multocida* infections display host specificity and/or disease predilection (2, 3, 134). Support for this notion comes primarily from epidemiological studies that show that isolates with certain capsular serogroups from disease outbreaks are associated with particular host species (134). However, the molecular basis for host specificity and/or disease predilection remains unclear. Several studies comparing the genomes of *P. multocida* isolates from different clinical presentations have provided some insights about their pathogenicity and host specificity (19, 21, 22, 135, 136), with some providing genomic evidence regarding disease predilection.

One study comparing the genomes of 13 isolates from bovine HS cases (12 Asian HS strains plus 1 North America HS strain) with 4 non-HS isolates from bovine (strain 36950), swine (strains 3480 and HN06), and poultry (strain Pm70) species identified more than 96 genes unique to the HS-associated strains (21). In another study, comparative genomic analysis of 23 *P. multocida* isolates associated with multiple types of diseases (pneumonia, progressive atrophic rhinitis, HS, fowl cholera, and snuffles) in different hosts (swine, bovine, poultry, alpaca, and rabbit) identified a number of

genomic islands and unique genes, namely, 127, 79, 18, and 155 genes, that are exclusively shared by the HS, fowl cholera, snuffles, and pneumonia isolates, respectively (135). A list of the genomic islands and unique genes is provided in Table S1 in the supplemental material. However, this study was unable to determine a specific discriminatory mechanism that could decisively correlate the presence of particular genes with their adaptation to a specific host/disease (135), so it may be necessary to await the availability of more genomes to determine this.

Recently, a larger-scale comparative genomic analysis was performed to explore the genomic basis for host predilection (19). In this study, the genome sequences of 109 *P. multocida* strains of different capsular serogroups (A, B, D, or F) and from different hosts (poultry, bovine, swine, or rabbit) with multiple types of diseases (fowl cholera, HS, progressive atrophic rhinitis, pneumonia, or snuffles) were compared using genotyping and pangenome analyses. While initial genotyping using WGS data supported the idea that *P. multocida* isolates from different hosts are associated with certain categories of capsular:LPS:MLST genotypes, the preferences displayed were not as strict as expected. For instance, many isolates from bovine species belong to type B:L2:ST122, as this genotype is frequently associated with bovine HS (21, 35, 47), but isolate HN04 from a swine HS case also belongs to the same genotype. Moreover, isolates with type F:L3:ST9 have been recovered from both avian species with fowl cholera and swine with pneumonia (16, 20). Likewise, this recent large comparative pangenomic analysis of *P. multocida* genomes (19) did not find any genes specific to a particular type of host, and virulence factor-associated genes did not show any host specificity as well. This pangenomic study also did not find any specific indispensable functional mechanism to decisively correlate the presence of genes and their adaptation to a specific host/disease. Therefore, other, as-yet-unidentified factors may be at play for determining host specialization.

Comparative genome analyses of *P. multocida* strains from different hosts have helped to decipher genetic divergence and pathogenic adaptation of the bacterium (22, 135, 136). Comparisons of the complete genomes of five *P. multocida* strains isolated from poultry (strain Pm70), swine (strains HB03, HN06, and 3480), and bovine (strain 36950) species, all causing diverse clinical presentations, revealed a number of sequence differences (22). These differences entailed a variety of missense mutations, nonsense mutations, and insertions-deletions, which could lead to alterations in the predicted proteins and their associated cellular components, such as the cell envelope and various metabolic processes, thereby impacting the pathogenicity and/or host adaptation of the bacterium. However, it should be noted that a small number of the observed mutations could be due to sequencing errors and/or annotation errors. Comparative genomics analysis of 33 different swine, bovine, leporine, avian, and human isolates of *P. multocida* identified genes that are subject to strong selective pressure, with the majority of positively selected genes coding for proteins localized to the cell envelope, suggesting that positive selection driven by host immune and defense systems acts predominantly on cell surface components or membrane structures (136).

PROPHAGES

In addition to the phage-related structural genes, bacterial genomes often harbor regions with prophage-associated genes that include satellite phage-like elements, called phage-inducible chromosomal islands (PICIs), that contribute to horizontal gene transfer, host adaptation, and virulence in many pathogens (137). Both prophages and their cognate PICIs occur in *P. multocida* genomes (17, 20, 21, 131, 133). PICIs are generally characterized as having (i) a well-conserved gene organization, including an integrase, a replicating module, and a master transcriptional regulator; (ii) unique attachment (*att*) sites that are never occupied by prophages; (iii) the absence of phage structural and lytic genes; and (iv) a size typically around 12 to 15 kb (137).

While little is known about the roles of PICIs in *Pasteurella* biology, these prophage-related sequences are found in strain-specific regions of many *P. multocida* genomes

TABLE 3 Predicted prophage genes/genomes in the complete genomes of representative *P. multocida* strains associated with different diseases in different hosts[a]

Strain	Host species	Disease	Capsular type	GenBank accession no.	No. of intact prophages	No. of incomplete prophages
3480	Swine	Pneumonia	A	CP001409	1	2
36950	Bovine	Pneumonia	A	CP003022	0	2
HB01	Bovine	Pneumonia	A	CP006976	3	0
HB03	Swine	Pneumonia	A	CP003328	1	0
HN06	Swine	Atrophic rhinitis	D	CP003313	3	1
HN07	Swine	Pneumonia	F	CP007040	0	1
P1059	Avian	Fowl cholera	A	CM001581	1	0
P1062	Bovine	Pneumonia	A	CM002276	6	2
X73	Avian	Fowl cholera	A	CM001580	0	1
Pm-3	Bovine	Pneumonia	A	CP014618	3	1
Razi_0001	Bovine	Hemorrhagic septicemia	B	CP017961	2	1
Razi_0002	Avian	Fowl cholera	A	CP019081	1	0
CIRMBP-0873	Rabbit	Snuffles	A	CP020347	5	0
CIRMBP-0884	Rabbit	Snuffles	A	CP020345	4	0
FDAARGOS_216	Avian	Fowl cholera	A	CP020403	0	1
FDAARGOS_218	Avian	Fowl cholera	A	CP020405	1	0
ATCC 43137	Swine	Pneumonia	A	CP008918	0	1

[a]Prophages were predicted by PHASTER.

(17, 20, 21, 131, 133) and, notably, are prevalent in genomes of *P. multocida* isolates associated with different clinical presentations in different hosts (Table 3). Several inducible lysogenic phages found in *P. multocida* genomes have been characterized (82, 137, 138).

A lambda-like phage belonging to the *Siphoviridae* family with a small isometric head of approximately 55 to 60 nm in diameter and a noncontractile tail carries the PMT-encoding gene *toxA* (82), which is present in the genomes of toxigenic *P. multocida* serogroup D strains (82). The life cycle of this lysogenic phage is believed to be responsible for the release of the toxin. The intact genomic sequence of this phage is not found in any of the currently sequenced *P. multocida* genomes excluding that of strain HN06, but a segment of this prophage sequence lacking the *toxA* gene was found in the genomes of some nontoxigenic isolates, including strains 3480, SH01 (GenBank accession no. NQMU01000000), SH02 (GenBank accession no. NWTL01000000), SH03 (GenBank accession no. NWTS01000000), SH04 (GenBank accession no. NWUD01000000), and SH05 (GenBank accession no. NWTT01000000) (Fig. 12). In addition, over 25 prophage-associated genes, including those encoding 14 Mu-like prophage proteins, have been identified as HS-specific genes (21). These findings strongly support the roles of these prophage-related areas in contributing to horizontal gene transfer and possibly influencing the short-term evolution of *P. multocida*, which in turn could be beneficial to host adaption and virulence (137).

A temperate bacteriophage, F108 (GenBank accession no. DQ114220), has been isolated from a type A strain through induction by mitomycin C, and its genome has been completely sequenced (138). The F108 virions displayed morphological characteristics similar to those of members of the *Myoviridae* family, with a hexagonal head measuring approximately 50 nm and a tail that is 120 nm long and 20 nm wide. The F108 genome was identified in the genomes of *P. multocida* strains CIRMBP-0873 and CIRMBP-0884, two type A isolates associated with snuffles in rabbits (135).

A Mu-like bacteriophage, AFS-2018a (GenBank accession no. MH238466), belonging to the *Myoviridae* family, has been isolated from *P. multocida* isolate Pm172 (137). This phage had a small, hexagonal head of approximately 38 to 39 nm long and 37 nm wide, attached to a 150-nm-long and 15-nm-wide tail, as determined by electron microscopy. AFS-2018a is also present in type B strain Pm86, and it was determined to be a bona fide PICI helper phage in *P. multocida* (137).

INTEGRATIVE AND CONJUGATIVE ELEMENTS

Integrative and conjugative elements (ICEs) are a class of mobile genetic elements capable of integrating into the bacterial chromosome via site-specific recombination

and encode a functional conjugation system, such as the T4SS, that mediates their transfer between bacterial cells (139). Functionally, ICEs often act as vehicles for the spread of antibiotic resistance and virulence factors (140–142). T4SSs commonly act as translocon machinery for a wide range of secreted toxic effector proteins, DNA and protein-DNA conjugation complexes, as well as assembly systems for bacterial surface pili or protein adhesins, thereby playing important roles in bacterial pathogenicity and host adaptation (143, 144).

Although ICEs are rarely detected in *P. multocida*, analysis of the complete genome sequence of a multidrug resistance strain, 36950, identified the first ICE in *P. multocida* (79). This 82-kb ICE, designated ICE*Pmu1*, is integrated into a $tRNA^{Leu}$ gene and is flanked by 13-bp direct repeats (80). ICE*Pmu1* contains 88 genes, including two regions of 15.7 and 9.8 kb that harbor 12 antibiotic resistance genes conferring resistance to streptomycin-spectinomycin (*aadA25*), streptomycin (*strA* and *strB*), gentamicin (*aadB*), kanamycin-neomycin (*aphA1*), tetracycline [*tetR-tet*(H)], chloramphenicol-florfenicol (*floR*), sulfonamides (*sul2*), tilmicosin-clindamycin [*erm*(42)], and tilmicosin-tulathromycin [*msr*(E)-*mph*(E)] (79, 80). In addition to the regions with resistance genes, ICE*Pmu1* possesses a number of insertion elements (IS*Apl1*, IS*CR21*, IS*CR2*, and IS*26*), suggesting that acquisition of resistance genes is associated with horizontal gene transfer events (79).

A second putative ICE, designated ICE*Pmu2*, in *P. multocida* was identified by analysis of the genome sequences of two HS-associated isolates, strains BUKK and Tx1 (21). ICE*Pmu2* carries at least 7 antibiotic resistance genes that confer resistance to aminoglycoside [*strA*, *strB*, and *aph(3′)-Ic*], β-lactam antibiotics (bla_{TEM-1B}), chloramphenicol (*catA2*), sulfonamides (*sul2*), and tetracycline [*tet*(H)]. ICE*Pmu2* may have a conjugative transfer ability similar to that of ICE*Pmu1* since genes identified in ICE*Pmu1* that might encode proteins involved in conjugative transfer are also found in ICE*Pmu2* (21), including a protein necessary for the formation of a type IV pilus, TraC, TraD, and TraG, as well as the tyrosine recombinases of the Xer family.

The putative ICE*pmcn05* found in the genome of the nontoxigenic strain HN05 encodes 59 proteins, some with predicted functions in excision/integration and conjugative transfer: a putative phage integrase (PMCN05_1005) showed similarity to two integrases found in ICE*Pmu1* within strain 36950 (identities of 41.72% and 51.55% with integrases Pmu_02700 and Pmu_02880, respectively) (80). These integrases share significant similarity with tyrosine recombinases of the Xer family, which mediate integration via site-specific recombination. A putative relaxase (PMCN05_1006, with 46.56% identity to Pmu_02890) is found downstream of the integrase gene; a similar organization is also found in ICE*Pmu1* (80). ICE*pmcn05* carries proteins necessary for the formation of a type IV pilus (PMCN05_1045, with 58.99% similarity to Pmu_03230) and for conjugative transfer, including TraD-like (PMCN05_1041, with 60.25% similarity to Pmu_03190), TraG-like (PMCN05_1038, with 47.82% similarity to Pmu_03040), and TraC-like (PMCN05_1030, with 56.02% similarity to Pmu_03070) proteins (21, 80). ICE*pmcn05* also encodes a putative DNA topoisomerase III protein, which is involved in DNA replication (PMCN05_1054, with 69.60% similarity to Pmu_03290); a protein with a lysozyme-like domain (PMCN05_1043, with 54.94% similarity to Pmu_03210); proteins involved in DNA replication, such as the single-stranded DNA-binding protein (PMCN05_1057, with 53.06% similarity to Pmu_03540), an ATPase involved in chromosome partitioning (PMCN05_1064, with 53.73% similarity to Pmu_03610), as well as a DNA helicase, DnaB (PMCN05_1063, with 43.88% similarity to Pmu_03600); and a ParB family protein (PMCN05_1062, with 41.39% similarity to Pmu_03590) that has a predicted DNA nuclease function (80).

An ICE, designated ICE*pmcn07*, has also been identified in the genome of a capsular type F isolate (HN07) from swine (20). ICE*pmcn07* is adjacent to but not integrated into a $tRNA^{Leu}$ gene. ICE*pmcn07* displays little homology to ICE*Pmu1* or ICE*Pmu2* but is highly homologous (>78% DNA identity) to an antibiotic resistance-conferring element, ICE*hin1056* (GenBank accession no. AJ627386), reported in *Haemophilus influenzae* (20). ICE*pmcn07* contains genes associated with tetracycline resistance as well as genes involved in replication and integration (Fig. 14). In particular, ICE*pmcn07* contains

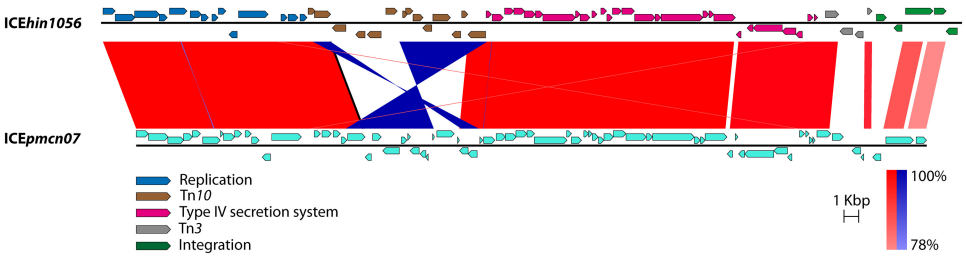

FIG 14 Comparative sequence analysis of ICE*hin1056* and ICE*pmcn07*. Shown is a synteny plot of ICE*hin1056* and ICE*pmcn07*, visualized by using EasyFig 2.2.3 (155). Arrows represent genes and their orientations within the genomic islands. Blocks with different color gradients show DNA identities between the two sequences. Arrows with the indicated colors refer to genes involved in different functions.

a complete set of genes encoding a putative T4SS (Fig. 14). The presence of this T4SS suggests that the mobility mechanism of ICE*pmcn07* might be different from that of ICEP*mu1* and ICEP*mu2*.

PASTEURELLA PLASMIDS

Several *P. multocida* isolates harbor plasmid genomes (81, 145), most of which carry antibiotic resistance genes. As of 31 December 2018, 18 sequences of *P. multocida* plasmids are publicly available through the NCBI genome database (Table 4). Of particular note is pIG1, a 5.36-kb plasmid isolated from toxigenic type D isolates associated with progressive atrophic rhinitis in Australia (145). The plasmid contains a 1.6-kb (bp 2284 to 3889) noncoding region responsible for plasmid replication, a 1.4-kb (bp 3890 to 5322) region responsible for plasmid mobilization (*mbeAy*, *mbeBy*, and *mbeCy*) that shares homology (99% similarity) to that of the *E. coli* plasmid ColE1, a 2.18-kb (bp 111 to 2283) region harboring genes conferring antibiotic resistance to sulfonamides (*sul2*) and streptomycin (*strA*), and a putative gene coding for a hypothetical protein of 79 amino acids whose N terminus has homology to the sulfonamide-resistant dihydropteroate synthase (Sul2) (145). Interestingly, an orthologous plasmid of pIG1, designated pHN06, was found in a toxigenic type D isolate associated with progressive atrophic rhinitis in China (81). pHN06 was 100% identical to pIG1 and shared the same genetic structure (Fig. 15).

The 1.4-kb mobilization area of pIG1 is also present in other *Pasteurella* plasmids, pB1018, pB1006, pB1005, pB1000, pOV, and pCCK411 (Fig. 15), suggesting that the mobilization of these plasmids relies on a mechanism similar to that of pIG1. The

TABLE 4 General features of sequenced *P. multocida* plasmids

Plasmid	GenBank accession no.	Size (kb)	% GC content	No. of genes	No. of proteins	No. of pseudogenes
pFDAARGOS_218	CP020404	3.742	41.8493	4	3	1
pB1005	FJ197818	4.237	48.1237	5	5	
pB1000	GU080062	4.613	41.7082	4	4	
pCCK647	AJ884726	5.198	42.6703	5	5	
pJR2	AY232671	5.252	41.8126	5	5	
pCCK411	FR798946	5.265	42.0703	5	5	
pIG1	U57647	5.36	47.4627	6	5	1
pHN06	CP003314	5.36	47.4813	7	6	1
pB1006	FJ234438	6.033	40.4442	4	4	
pB1018	JQ319774	6.074	39.1999	5	5	
pJR1	AY232670	6.792	45.6567	6	6	
pCCK1900	FM179941	10.226	60.6787	9	9	
pCCK381	AJ871969	10.874	60.6676	6	5	1
pOV	JX827416	13.551	39.7019	16	16	
pCIRMBP-0873-2	CP020349	28.093	41.2736	27	3	20
pS298D	CM009574	29.269	31.067	33	32	1
pCIRMBP-0873-1	CP020348	34.596	41.5828	35	7	24
pCIRMBP-0884	CP020346	325.255	40.1716	372	324	29

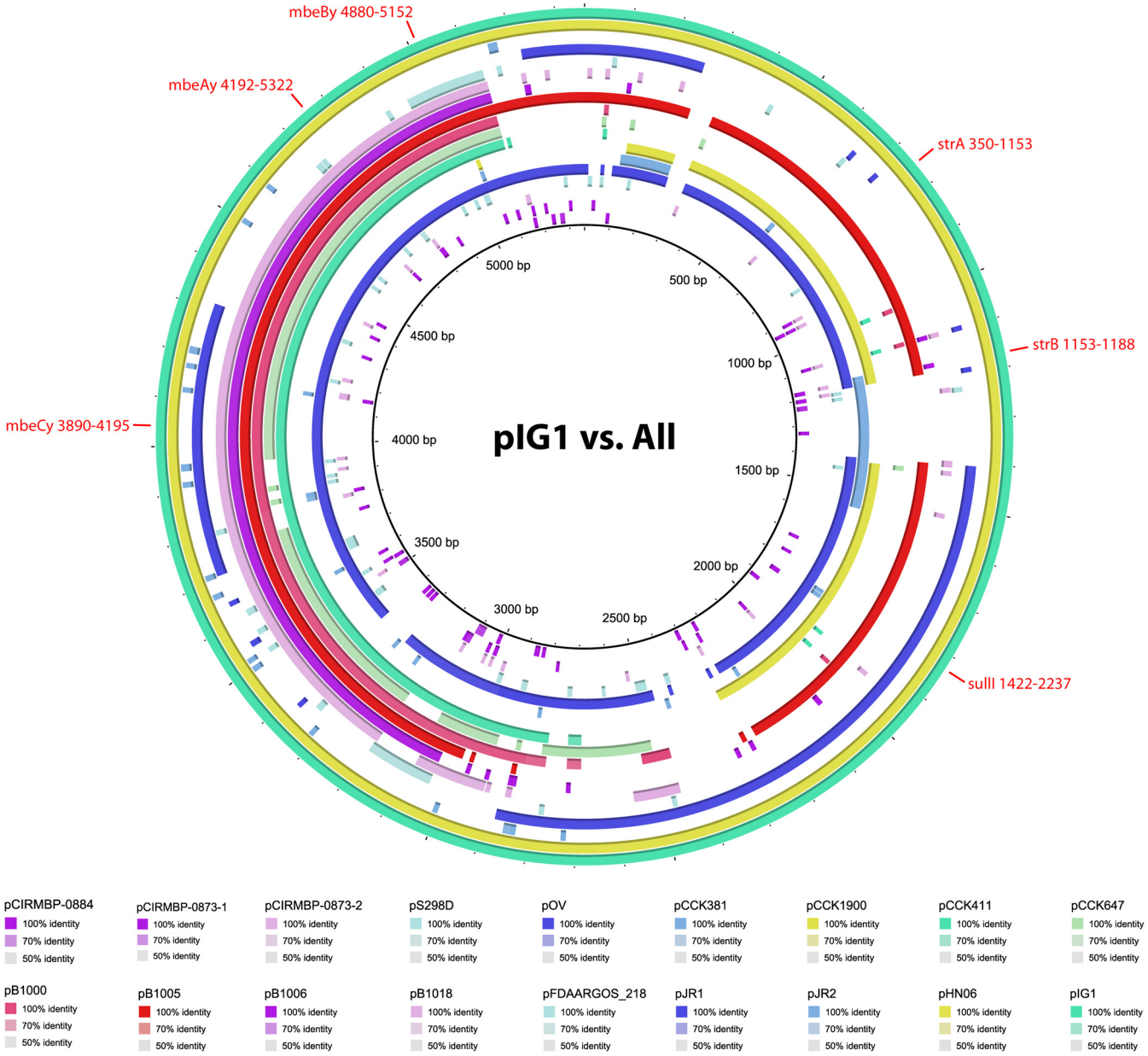

FIG 15 Comparative genomics of sequenced *Pasteurella* plasmids. The genome sequences that were compared using the BRIG package (160) included the plasmids listed in Table 4. DNA identities between each of the genome sequences are shown. The functions of the regions displaying low identities between different genome sequences are marked as bars at the outermost circle.

plasmids pB1000, pB1005, and pB1006 belong to the ColE1 superfamily and were isolated from porcine isolates resistant to multiple antibiotics (146). Analysis of the plasmid sequences identified a β-lactam (bla_{ROB-1}) resistance gene on pB1000, sulfonamide (*sul2*) and streptomycin (*strA*) resistance genes on pB1005 and pB1006, and a tetracycline [*tet*(O)] resistance gene on pB1006. It is of note that pB1000, pB1005, and pB1006 contain a putative *oriV* region upstream of the mobilization genes, predicted to be responsible for plasmid replication and copy number control (146). Interestingly, this *oriV* region is also present in pIG1 and pHN06 (Fig. 15).

Some plasmids do not contain the ColE1-like mobilization genes (Fig. 15). Two such plasmids, designated pJR1 (6.79 kb) and pJR2 (5.25 kb), were isolated from isolates associated with fowl cholera outbreaks in Taiwan (147). The plasmid pJR1 contains three genes conferring resistance to sulfonamides (*sul2*), tetracycline (*tetG*), and chlor-

amphenicol (*catB2*), while the plasmid pJR2 carries genes conferring resistance to streptomycin and spectinomycin (*aadA1*) and ampicillin and carbenicillin (*blaP1*). Another two such plasmids, designated pCCK1900 (10.2 kb) and pCCK381 (10.9 kb), were obtained from florfenicol-resistant *P. multocida* isolates (148, 149). Both plasmids contain a *floR* gene responsible for the florfenicol-resistant phenotype; this gene also confers resistance to chloramphenicol. In addition, pCCK1900 contains genes conferring resistance to streptomycin (*strA* and *strB*) and sulfonamides (*sul2*) (149). It is of note that pCCK381 contains a mobilization region similar to that of pDN1 from *Dichelobacter nodosus* (148, 150), while pCCK1900 contains a mobilization region homologous to that of the broad-host-range plasmid RSF1010 (149, 151).

PASTEURELLA MULTOCIDA PHYLOGENY

Comparative genome sequence analysis using strain Pm70 suggests that *P. multocida* diverged from *H. influenzae* about 270 million years ago (mya) (range of 138 to 407 mya) and shares a common ancestor with *E. coli* about 680 mya (range of 319 to 1,024 mya) (23). Phylogenetic relationships among different *P. multocida* isolates, based on 16S rRNA genes, MLST genes, whole-genome sequences, single nucleotide variants (SNVs) across the whole-genome sequence, and core genome SNVs, have been examined using a variety of methods and bioinformatics tools, such as MEGA, SplitsTree, and FastTree2 (21, 22, 63, 135, 136). However, as mentioned above, none of these studies to date have successfully separated *P. multocida* isolates according to serotype, disease, place of isolation, and/or host of isolation, so it was concluded that little or no correlation exists between the phylogenetic relatedness of *P. multocida* strains and any of these metadata (133, 136). However, it is possible that comparisons using more isolates of each type are needed for discrimination.

A recent comparative phylogenetic analysis compared either SNVs or core genome SNVs within the whole genomes from avian, bovine, porcine, or leporine isolates of *P. multocida* to uncover genetic traits associated with host specificity (19). Results revealed that strains with the same LPS genotype plus MLST genotype clustered together (Fig. 16). While additional *P. multocida* genome sequences are needed to determine whether this phylogenetic relationship trend holds, these findings suggest that combining information from both the LPS typing and MLST methods might provide more discriminatory power for assessing factors that contribute to *P. multocida* phylogeny.

More recent bioinformatics and Bayesian analytical tools based on whole-genome SNVs, such as that available in the BEAST2 package (152, 153), enable the application of WGS data for comparative epidemiology and molecular evolutionary studies by also allowing the inclusion of demographic information, such as capsular and LPS genotyping; the presence of adhesins, toxins, or other virulence factors; metabolic typing; timing of isolation; geographic location; host source; and other metadata. An interesting example of the power of these types of analytics is a recent report of the evolutionary history of the ST131 lineage of multidrug-resistant *Escherichia coli* (154), where chromosomal phylogenetic comparisons among a large number of ST131 strains using BEAST2 enabled more refined sequence typing along with geographic and time-scaled clonal tracking. While the application of such methods is not yet practical for tracking *P. multocida* lineages due to the limited number of currently available genome sequences, these types of analyses could provide important evolutionary insights for future studies of this important group of bacteria.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Although completion of genome sequences for *P. multocida* isolates has lagged behind those of many other Gram-negative pathogenic bacteria, the recent availability of genome sequences from multiple strain types of *P. multocida* has already significantly advanced our understanding of the pathogenesis of *P. multocida*. However, there is still a long way to go before we fully understand the pathogenic mechanisms of this important zoonotic pathogen. Many more *P. multocida* genomes, especially for isolates from uncommon host species, are needed for further consideration. In addition, until

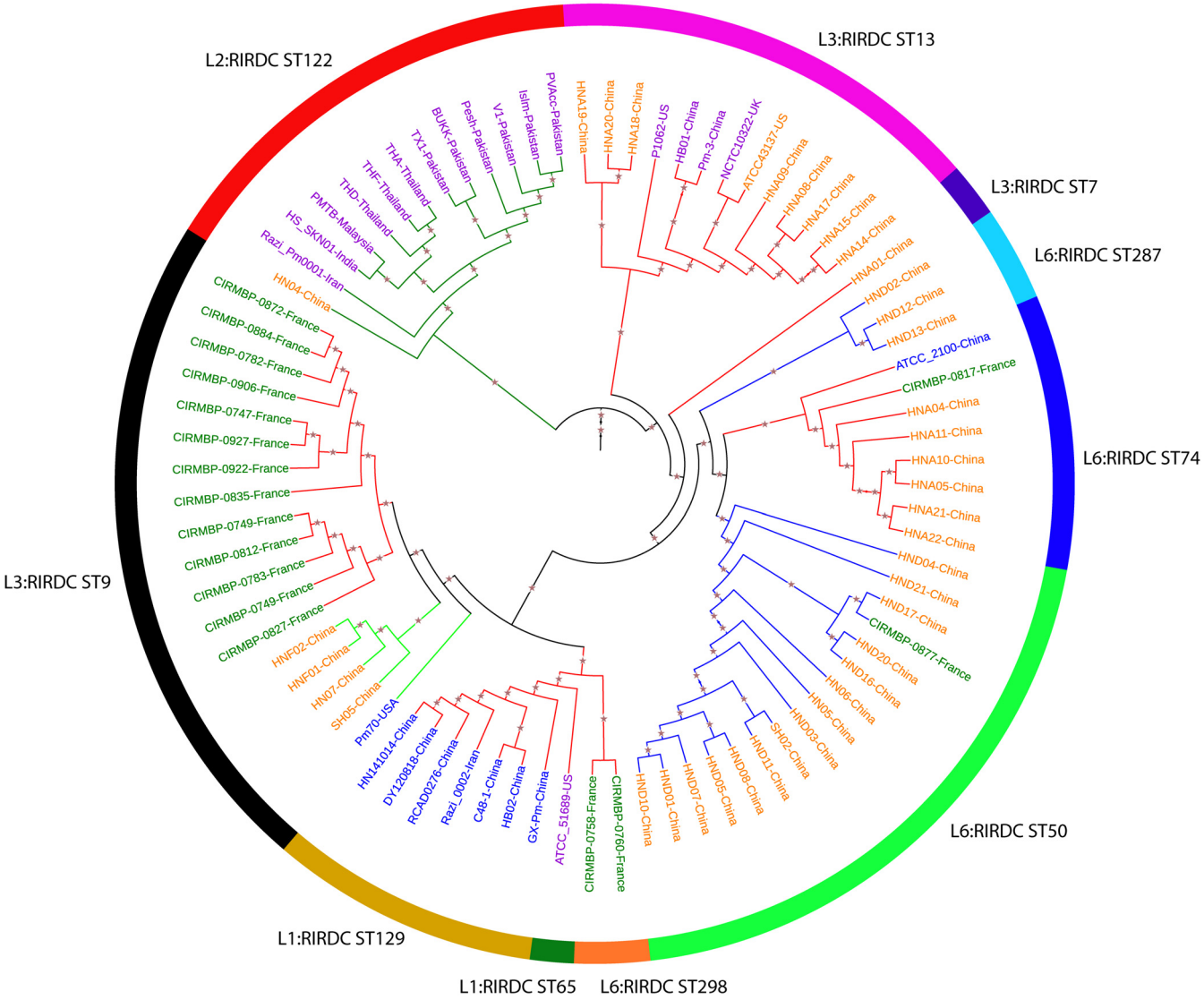

FIG 16 Phylogenetic relationship tree of selected *P. multocida* isolates with different serotypes/genotypes from different host species. The tree was generated using single nucleotide variants across the whole-genome sequences of 82 *P. multocida* strains (accession numbers are given in Table S2 in the supplemental material). Strains of different capsular serotypes/genotypes are located on branches and denoted by different colors (red, type A; dark green, type B; blue, type D; light green, type F). Strains from different hosts are marked in different colors (blue, avian isolates; purple, bovine isolates; orange, porcine isolates; dark green, leporine isolates). The outmost ring gives the LPS:MLST genotypes of each clade. The stars denote bootstrap values within the range of 0.103 to 1.000.

third-generation sequencing technologies, such as PacBio and/or Nanopore methods, become more affordable, accurate, and reliable, a combination of the second-generation with third-generation sequencing technologies should be considered for future *P. multocida* genome sequencing projects, as this strategy could improve the quality of sequencing data and ease of assembly. Finally, improved mutagenesis tools are required to experimentally confirm the functions of the virulence- and/or fitness-associated genes/gene clusters that have been identified through comparative genome sequencing analysis.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/MMBR.00014-19.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.2 MB.

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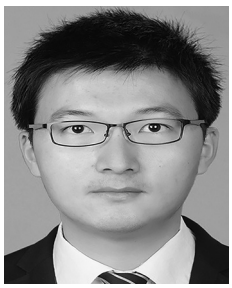

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Rui Zhou leads a research group whose research focuses on microbial genomics and pathogenesis of bacterial infectious diseases that threaten farm animal and human health. He obtained a bachelor's degree in Veterinary Medicine in 1993 and a master's degree in Veterinary Pathology in 1996, both at HZAU, China. In 2003, he obtained his Ph.D. in Veterinary Medicine at the University of Munich, Germany. He was appointed Full Professor at HZAU in 2007. The majority of Dr. Zhou's work has been in *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, and *Staphylococcus aureus*. Research interests include epidemiology and antibiotic resistance, bacterial genomics, mechanisms of bacterial cell division, host-bacterium interactions, and diagnosis and vaccines.

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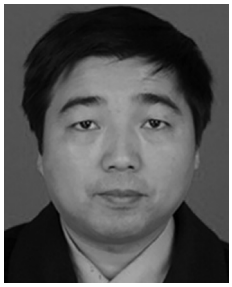

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